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THE BIOCHEMICAL JOURNAL

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CONTENTS

No. 1

PAGE

I. The effect of heat upon the activating efficiency of enterokinase. By J. Pace. (With two figures) 1

II. On the quantity of free water in the red blood corpuscles. By E. Schiødt 8

III. Hydroxypyruvic aldehyde: its preparation and physiological behaviour. By A. Hynd 11

IV. Spectrographic data concerning vitamin A and liver oils. By R. A. Morton, I. M. Heilbron and A. Thompson. (With seven figures) . . 20

V. Spectrographic data of natural fats and their fatty acids in relation to vitamin A. By A. E. Gillam, I. M. Heilbron, T. P. Hilditch and R. A. Morton. (With nine figures) 30

VI. Studies in the biochemistry of micro-organisms. XIX. 6-Hydroxy-2-methylbenzoic acid, a product of the metabolism of glucose by *Penicillium griseo-fulvum* Dierckx. By W. K. Anslow and H. Raistrick . . . 39

VII. On the behaviour of "indifferent" electrodes when used for the determination of oxidation-reduction potentials in the presence of hydrogen. By E. H. Lepper and C. J. Martin. (With four figures) 45

VIII. The fatty acids of the cat's kidney. I. By K. Turner . . . 49

IX. The permeability of human epidermis to ultra-violet irradiation. By N. S. Lucas. (With seven figures) 57

X. The relation between cystine yield and total sulphur in kemp and outer-coat animal fibres. By C. Rimington 71

XI. Colour reactions of sterols with nitric acid. By O. Rosenheim and R. K. Callow 74

XII. The purification of ergosterol. By R. K. Callow. (With one figure) 79

XIII. Occurrence of α-dihydroergosterol as an impurity in yeast ergosterol. By R. K. Callow 87

XIV. Some peculiarities in the glyceride structure of laurel fats. By G. Collin 95

XV. Iodimetric determination of reducing sugars in the apple. By H. K. Archbold and E. M. Widdowson 101

XVI. Biological oxidations in the succinic acid series. By J. H. Quastel and A. H. M. Wheatley 117

XVII. A study of the mechanism of the degradation of citric acid by *B. pyocyaneus* (*Pseudomonas pyocyanea*). II. Action of *B. pyocyaneus* on succinic acid. By T. K. Walker, V. Subramaniam, H. B. Stent and J. Butterworth 129

XVIII. The antiscorbutic potency of apples. II. By M. F. Bracewell, F. Kidd, C. West and S. S. Zilva 138

XIX. The antiscorbutic potency of apples. III. By M. F. Bracewell, T. Wallace and S. S. Zilva 144

XX. The biological oxidation of carbohydrate solutions. Part I. The oxidation of sucrose and ammonia in sectional percolating filters. By S. H. Jenkins. (With one figure) 147

PAGE

XXI. Notes on the Hagedorn-Jensen method for the determination of blood-sugar. By H. KRAMER and A. STEINER 161

XXII. The vapour pressure depressions of aqueous solutions of phosphate buffer mixtures at 20·3°. By A. GROLLMAN. (With one figure) . . . 166

XXIII. Oxidation-reduction potentials of pneumococcus cultures. II. Effect of catalase. By L. F. HEWITT. (With six figures) 169

XXIV. The temperature coefficients and energy exchanges of the citric acid dehydrogenase of cucumber seeds. By W. J. DANN. (With two figures) . 177

XXV. The amino-acids of glutenin. By M. DAMODARAN . . . 190

XXVI. Studies on the gums. II. Tragacanthin—the soluble constituent of gum tragacanth. By A. G. NORMAN. (With one figure) 200

XXVII. Hydrogenase: a bacterial enzyme activating molecular hydrogen. I. The properties of the enzyme. By M. STEPHENSON and L. H. STICKLAND (With five figures) 205

XXVIII. Hydrogenase. II. The reduction of sulphate to sulphide by molecular hydrogen. By M. STEPHENSON and L. H. STICKLAND. (With two figures) 215

XXIX. The effect of insulin on growth, nitrogen excretion and respiratory metabolism. By M. W. GOLDBLATT and R. W. B. ELLIS 221

XXX. The distribution of nitrogen and sulphur in the urine during conditions of increased catabolism. By D. P. CUTHBERTSON 236

XXXI. Selective fermentation. Alcholic fermentation of mixtures of glucose and fructose by brewer's and Sauterne yeasts. By R. H. HOPKINS . 245

XXXII. The proteolytic enzymes of green malt. II. Action of the proteinase on egg-albumin, caseinogen, edestin and fibrin at different reactions. By R. H. HOPKINS and H. E. KELLY 256

XXXIII. Carotene and vitamin A. The conversion of carotene into vitamin A by fowl. By N. S. CAPPER, I. M. W. MCKIBBIN and J. H. PRENTICE. (With five figures) 265

XXXIV. Vitamin A and carotene. VII. The distribution of vitamin A and carotene in the body of the rat. By T. MOORE. (With two figures) . 275

XXXV. The biochemistry of aluminium. II. Excretion and absorption of aluminium in the rat. By K. MACKENZIE. (With one figure) . . . 287

XXXVI. Calcification of the bones of rats on a diet low in ergosterol. By E. M. HUME and H. H. SMITH 292

XXXVII. The relation of a fat-free diet to the scaly tail condition in rats described by Burr and Burr. By E. M. HUME and H. H. SMITH . . . 300

XXXVIII. The micro-determination of barium. By R. R. MCLAUGHLIN 307

XXXIX. The methylation of hexosemonophosphoric ester. By E. J. KING, R. R. MCLAUGHLIN and W. T. J. MORGAN. (With three figures) . . 310

XL. Hexosemonophosphoric esters. By R. ROBISON and E. J. KING. (With one figure) 323

XLI. Studies in blood glycolysis. Preliminary observations. By C. REID and B. NARAYANA 339

XLII. The addition compound theory of enzyme action. By B. WOOLF . 342

XLIII. On the inadequacy of white wheat flour and fish meal for prolonged growth of pigs and its amelioration with yeast or stout. By A. H. BLISSETT and J. GOLDING. (With two figures) 349

XLIV. The fat-soluble vitamin requirements of the chick. I. The vitamin A and vitamin D content of fish meal and meat meal. By W. D. MCFARLANE, W. R. GRAHAM, Jr., and F. RICHARDSON. (With four figures) 358

PAGE

XLV. The mode of action of vitamin D. Studies on hypervitaminosis D. The influence of the calcium-phosphate intake. By L. J. Harris and J. R. M. Innes. (With Plates I and II and fifteen figures) 367

XLVI. The electrophoretic velocities of gelatin and ovalbumin in different concentrations of their mixtures and the effect of ultra-violet irradiation. By E. B. R. Prideaux and F. O. Howitt. (With two figures) 391

No. 2

XLVII. On the nature of the cell wall constituents of *Laminaria* spp. Mannuronic acid. By G. M. Bird and P. Haas 403

XLVIII. Note on dihydroxyacetone. By H. G. Reeves and E. T. Renbom 412

XLIX. The effect of arginine upon the body weight of mice injected with thyroxine, and bearing the tumour M 63. By E. Gilroy 414

L. Is avitaminosis B_1 an intoxication by methylglyoxal? Glyoxalase-co-enzyme ratio in experimental beriberi. By P. Vogt-Møller . . . 418

LI. The inactivation of trypsin-kinase by heat, and the effect of added protein thereon. By J. Pace. (With one figure) 422

LII. The molecular weight of vitamin A. By H. R. Bruins, J. Overhoff and L. K. Wolff 430

LIII. Intermediary carbohydrate metabolism. The effect of sodium iodoacetate on glyoxalase. By H. W. Dudley. (With one figure) . . 439

LIV. A further note on the determination of iodine in blood. By H. A. A. Aitken. (With one figure) 446

LV. The colorimetric determination of sodium. By R. A. McCance and H. L. Shipp. (With one figure) 449

LVI. Studies on the metabolism of animals on a carbohydrate-free diet. II. Variations in the sensitivity towards insulin of different species of animals on carbohydrate-free diets. By A. Hynd and D. L. Rotter . . . 457

LVII. Effect of the addition of salts on the p_H of solutions of ampholytes. By W. Giedroyć and S. J. Pryzłęcki 465

LVIII. The self-selection of food constituents by the rat. By S. K. Kon. (With four figures) 473

LIX. A study of the nitrogen balance in vitamin B_2 deficiency in the rat. By S. K. Kon. (With one figure) 482

LX. The biochemistry of dry-rot in wood. II. An investigation of the products of decay of spruce wood rotted by *Merulius lachrymans*. By E. C. Barton-Wright and J. G. Boswell 494

LXI. The glyceride structure of butter-fats. By T. P. Hilditch and J. J. Sleightholme. (With one figure) 507

LXII. The effect of hydrogen ion concentration on some oxidations by *B. coli communis*. By R. P. Cook and R. S. Alcock. (With seven figures) . 523

LXIII. The relationship between the respiratory catalysts of *B. coli*. By R. P. Cook, J. B. S. Haldane and L. W. Mapson. (With eleven figures) . 534

LXIV. Caseinogen for vitamin tests. By K. H. Coward, K. M. Key, F. J. Dyer and B. G. E. Morgan. (With one figure) 551

LXV. The relation between amylase and lactic acid formation in muscle. By E. M. Case 561

LXVI. The effect of starvation, phloridzin, thyroid, adrenaline, insulin and pituitrin on the distribution of glycogen in the rat. By R. D. Lawrence and R. A. McCance 570

PAGE

LXVII. Metabolism in scurvy. III. The absorption and retention of calcium and phosphorus by guinea-pigs. By F. E. Humphreys and S. S. Zilva 579

LXVIII. A note on the reprecipitation of the antiscorbutic factor from decitrated lemon juice. By S. S. Zilva 594

LXIX. The effect of desiccation upon the nutritive properties of egg-white. II. By M. A. B. Fixsen. (With two figures) 596

LXX. The nature of the lipoid matter extracted from green leaves (spinach and cabbage). By D. L. Collison and I. Smedley-MacLean . . 606

LXXI. The cuprous derivatives of some sulphydryl compounds. By N. W. Pirie 614

LXXII. The action of dyestuffs on enzymes. I. Dyestuffs and oxidations. By J. H. Quastel and A. H. M. Wheatley 629

LXXIII. The effect of adding vitamin A to a rachitogenic diet. By A. L. Bacharach, E. Allchorne and V. Hazley. (With one figure) . . . 639

LXXIV. The influence of calcium on the rate of diffusion of sugars through surviving intestine. By H. E. Magee and K. C. Sen. (With two figures) . 643

LXXV. On the influence of serum on enzymes, with special reference to its action on trypsin. By J. Fine. (With seventeen figures) . . . 647

LXXVI. The total carbohydrate content of isolated frog muscle. By M. Kerly. (With two figures) 671

No. 3

LXXVII. Experiments on nutrition. X. Comparative vitamin B_1 values of foodstuffs. Cereals II. By R. H. A. Plimmer, W. H. Raymond and J. Lowndes 691

LXXVIII. Structure and enzyme reactions. IX. The systems amylase-starch-gelatin and urease-urea-gelatin. By S. Freiberger 705

LXXIX. Effect of addition of salts on the isoelectric point of proteins. I. By S. J. Przyłęcki 713

LXXX. The phosphatase activity of transplants of the epithelium of the urinary bladder to the abdominal wall producing heterotopic ossification. By C. B. Huggins 728

LXXXI. Fat metabolism in muscular exercise. By C. P. Stewart, R. Gaddie and D. M. Dunlop. (With five figures) 733

LXXXII. The nature of tyrosinase. By D. Narayanamurti and C. V. Ramaswami 749

LXXXIII. The action of tannase on gallotannin. By W. N. Nicholson, M. Nierenstein, J. C. Pool and N. V. Price 752

LXXXIV. The chemical aspect of the drying of timber. 2. The drying of a softwood. By W. G. Campbell and J. Booth 756

LXXXV. A study of the hydrogen ion concentration of the potato tuber. By I. M. Robertson and A. M. Smith 763

LXXXVI. A note on the inhibitory effect of monoiodoacetic acid on lactic acid production by cancer tissue. By S. T. Harrison and E. Mellanby 770

LXXXVII. The blackening of potatoes after cooking. By C. K. Tinkler. (With Plate III) 773

LXXXVIII. Cyclic changes in the glycogen content of the liver and the muscles of rats and mice. Their bearing upon the sensitivity of the animals to insulin, and their influence on the urinary output of nitrogen. By G. Ågren, O. Wilander and E. Jorpes. (With six figures) 777

PAGE

LXXXIX. Metabolism studies in tetany. By S. Morris, A. McL. Watson and N. Morris 786

XC. The enzymic hydrolysis of lecithin. By E. J. King. (With two figures) 799

XCI. The nature of the "ether reaction" of urine. By W. J. Boyd . 812

XCII. Note on the reactivation of reductase in washed yeast preparations. By A. Harden and M. G. Macfarlane 818

XCIII. Fermentation by yeast preparations. I. The effect of mono-iodoacetate on the fermentation of hexosediphosphate. II. The action of arsenate on the induction period of zymin. By M. G. Macfarlane. (With four figures) 822

XCIV. The effect of hydrogen ion concentration on the precipitating and protective actions of proteins on colloidal gold and gum benzoin. By R. H. Hopkins, W. O. Kermack and W. T. Spragg. (With ten figures) . . . 829

XCV. Studies in the metabolism of tissues growing *in vitro*. IV. Effect of fructose, galactose and xylose upon the ammonia and urea production of embryo kidney tissue. By E. Watchorn and B. E. Holmes . . . 843

XCVI. The effect of narcotics on some dehydrogenases. By K. C. Sen. (With fourteen figures) 849

XCVII. Oxidation-reduction potentials of haemolytic streptococci. II. Effect of catalase. By L. F. Hewitt. (With two figures) . . . 858

XCVIII. A method for the determination of small quantities of mixed reducing sugars and its application to the estimation of the products of hydrolysis of starch by taka-diastase. By E. M. Widdowson . . . 863

XCIX. The respiration of *B. coli communis*. By R. P. Cook and J. B. S. Haldane. (With six figures) 880

C. A quantitative method for the determination of vitamin C. By K. M. Key and G. K. Elphick. (With Plates IV and V and two figures) . . 888

CI. The action of dyestuffs on enzymes. II. Fumarase. By J. H. Quastel 898

CII. The effect of vitamin B deficiency upon the vitamin A reserves of the rat. By W. J. Dann and T. Moore 914

CIII. The kinetics of peroxidase action. By P. J. G. Mann. (With eight figures) 918

CIV. Investigations on the preparation and behaviour of vitamin B_1 concentrates from yeast. By B. C. Guha. (With six figures) . . . 931

CV. Investigations on vitamin B_2. I. The sources of vitamin B_2. II. The stability of vitamin B_2. III. The chemistry of vitamin B_2. By B. C. Guha. (With nine figures) 945

CVI. Observations on certain factors necessary for the normal nutrition of the rat. By B. C. Guha. (With six figures) 960

No. 4

CVII. The metabolism of normal and tumour tissue. III. A method for the measurement of respiratory quotient in serum. By F. Dickens and F. Simer. (With one figure) 973

CVIII. The metabolism of normal and tumour tissue. IV. The respiratory quotient in bicarbonate-media. By F. Dickens and F. Simer . . . 985

CIX. Growth and chemical composition of ovum of functioning fowl's ovary (*Gallus domesticus*). By A. L. Romanoff 994

PAGE

CX. A new synthesis of methionine. By G. Barger and T. E. Weichselbaum 997

CXI. The vitamin D problem. II. Optical rotation of vitamin D. By E. H. Reerink and A. van Wijk. (With three figures) 1001

CXII. The oxidation of hexosediphosphoric acid by an enzyme from animal tissues. By D. C. Harrison 1011

CXIII. Glucose dehydrogenase: a new oxidising enzyme from animal tissues. By D. C. Harrison. (With two figures) 1016

CXIV. Synthesis of the *d*- and *l*-3 : 4-dihydroxyphenylalanines. By C. R. Harington and S. S. Randall 1028

CXV. The isolation of *d*-3 : 5-diiodotyrosine from the thyroid gland by the action of proteolytic enzymes. By C. R. Harington and S. S. Randall . 1032

CXVI. The metabolism of iodobenzene and *p*-iodophenylmercapturic acid in the rat and the rabbit. By N. R. Lawrie. (With three figures) . . 1037

CXVII. Observations on some alcohol-soluble proteins from milk products. By L. A. Allen 1045

CXVIII. The ferricyanide method for the determination of reducing sugars. A modification of the Hagedorn-Jensen-Hanes technique. By A. C. Hulme and R. Narain. (With two figures) 1051

CXIX. The carbohydrate complex of the serum proteins. II. Improved method for isolation and re-determination of structure. Isolation of glucosaminodimannose from proteins of ox blood. By C. Rimington . . . 1062

CXX. The relation between total sulphur and cystine content of certain samples of rabbit fur. By J. Barritt and C. Rimington 1072

CXXI. Note on the sulphur-free nature of medulla in blackface wool. By J. Barritt and A. T. King 1075

CXXII. Sulphur distribution in the component structures of wool and porcupine quills. By J. G. Bekker and A. T. King. (With one figure) . . 1077

CXXIII. Vitamin C in the orange and the grape fruit. By M. F. Bracewell and S. S. Zilva 1081

CXXIV. The chlorides and inorganic constituents of the serum and cerebrospinal fluid in nephritis and acidosis. By G. C. Linder and E. A. Carmichael. (With one figure) 1090

CXXV. Studies in the sulphur metabolism of the dog. VIII. The metabolism of glutathione compared with that of other cystine derivatives. By T. S. Hele and N. W. Pirie. (With three figures) 1095

CXXVI. The determination of vitamin A in cod-liver oils (*a*) biologically, (*b*) chemically, (*c*) physically, with a statistical examination of the results. By K. H. Coward, F. J. Dyer, R. A. Morton and J. H. Gaddum . . . 1102

CXXVII. Trypanocidal action and toxicity to enzymes. By J. H. Quastel 1121

CXXVIII. The relation of glycosuria to glycaemia and the determination of the renal threshold for glucose. By H. P. Himsworth. (With eleven figures) 1128

CXXIX. Studies on the relationship between chemical constitution and physiological action. III. The inhibitory action of certain synthetic urethanes on the activity of liver esterase. By E. Stedman and E. Stedman . . . 1147

CXXX. The glyceride structure of beef tallows. By A. Banks and T. P. Hilditch. (With one figure) 1168

CXXXI. Determination of inorganic sulphate in human blood-plasma by micro-titration. By C. L. Cope. (With one figure) 1183

CXXXII. The iodine method for the determination of glutathione. By N. Gavrilescu 1190

PAGE

CXXXIII. The fate of carotene after absorption in the animal organism. By B. AHMAD. (With one figure) 1195

CXXXIV. The distribution of the vitamin B complex. II. Root vegetables. By M. H. ROSCOE 1205

CXXXV. A differential resistance thermometer for biological use. By D. R. MCCULLAGH and E. M. CASE. (With five figures) 1213

CXXXVI. Studies on "non-irritable" muscle. By D. R. MCCULLAGH and E. M. CASE 1220

CXXXVII. The Erlenmeyer synthesis of amino-acids. By J. LAMB and W. ROBSON 1231

CXXXVIII. A preliminary note on the inorganic sulphate content of the blood with a method for its determination. By D. P. CUTHBERTSON and S. L. TOMPSETT. (With one figure) 1237

CXXXIX. The sensitisation of red blood corpuscles in haemolysis. By K. C. SEN. (With six figures) 1244

CXL. The oxidation of oleic acid by means of hydrogen peroxide with and without the addition of copper sulphate: a possible analogy with its oxidation *in vivo*. By I. SMEDLEY-MACLEAN and M. S. B. PEARCE 1252

CXLI. Some biochemical and physiological aspects of copper in animal nutrition. By I. J. CUNNINGHAM. (With five figures) 1267

CXLII. On the fundamental nature of vitamin D action. By J. P. MCGOWAN, I. J. CUNNINGHAM and D. W. AUCHINACHIE 1295

CXLIII. Studies on hemicelluloses. IV. The proximate analysis of boxwood, and the nature of its furfuraldehyde-yielding constituents. By I. A. PREECE 1304

CXLIV. The occurrence of pyruvic acid and methylglyoxal in muscle metabolism. By E. M. CASE and R. P. COOK 1319

CXLV. Pigmentation of the liver of the monk (or angler) fish. By J. A. LOVERN and R. A. MORTON. (With one figure) 1336

CXLVI. The Mittelmann process for treating fish-livers. By J. A. LOVERN, R. H. CREED and R. A. MORTON 1341

CXLVII. The antimony trichloride colour test and the ultra-violet absorption of liver oils and concentrates. By A. E. GILLAM and R. A. MORTON 1346

CXLVIII. Specificity in tests for vitamin A. A new conception of the chromogenic constituents of fresh and aged liver oils. By I. M. HEILBRON, A. E. GILLAM and R. A. MORTON. (With Plate VI and six figures) . . 1352

CXLIX. The physiological function of vitamin B_1. By B. C. GUHA. (With eleven figures) 1367

CL. On galactose as the dietary carbohydrate. By B. C. GUHA. (With five figures) 1385

CLI. The chemical nature of vitamin B_1 from evidence afforded by its electrical transference. By T. W. BIRCH and B. C. GUHA. (With five figures) 1391

CLII. Biochemical lesions in vitamin B deficiency. By N. GAVRILESCU and R. A. PETERS. (With two figures) 1397

CLIII. Carbohydrate metabolism in birds. III. The effects of rest and exercise upon the lactic acid content of the organs of normal and rice-fed pigeons. By R. B. FISHER. (With two figures) 1410

CLIV. The biological filtration of dilute sucrose solutions. By N. W. BARRITT. (With six figures) 1419

No. 5

PAGE

CLV. Effect of bacteriophage on oxidation-reduction potentials of *B. dysenteriae* (Shiga) cultures. By L. F. Hewitt. (With five figures) . 1447

CLVI. Effect of lysozyme on the oxidation-reduction potentials of *M. lysodeikticus* cultures. By L. F. Hewitt. (With three figures) . . 1452

CLVII. On an enzyme from blow-fly larvae [*Lucilia sericata*] which digests collagen in alkaline solution. By R. P. Hobson. (With one figure) . . 1458

CLVIII. The occurrence of vitamin D in lampreys (Petromyzontidae). By R. K. Callow and C. F. Fischmann 1464

CLIX. The occurrence of sugar alcohols in marine algae. Dulcitol. By P. Haas and T. G. Hill 1470

CLX. A preliminary note on the nitrogen metabolism of seaweeds. Glutamic acid peptide. By P. Haas and T. G. Hill 1472

CLXI. The biological values of the proteins of breads baked from rye and wheat flours alone or combined with yeast or soya bean flour. By S. K. Kon and Z. Markuze 1476

CLXII. The heat-inactivation of the proteinase of the pancreas. By J. Pace 1485

CLXIII. Autolysis in malignant and normal rabbit tissues. By H. I. Price 1491

CLXIV. The acid-denaturation of haemoglobin. By N. U. Meldrum. (With six figures) 1498

CLXV. Studies in the biochemistry of micro-organisms. XX. On the production of mannitol from hexoses and pentoses by a white species of *Aspergillus*. By F. P. Coyne and H. Raistrick 1513

CLXVI. Studies in the biochemistry of micro-organisms. XXI. Quantitative examination by the carbon balance sheet method of the types of products formed from glucose by species of bacteria. By J. H. Birkinshaw, J. H. V. Charles and P. W. Clutterbuck 1522

CLXVII. On the glyoxalase-co-enzyme ratio in liver tissue in inanition. By P. Vogt-Møller 1540

CLXVIII. The reduction of nitrates by *Bact. coli*. By L. H. Stickland. (With six figures) 1543

CLXIX. Structure and enzyme reactions. X. Action of salts on the systems: amylase-starch-proteins. By H. Chrempińska 1555

CLXX. The oxidation of sulphydryl compounds by hydrogen peroxide. I. Catalysis of oxidation of cysteine and glutathione by iron and copper. By N. W. Pirie. (With fourteen figures) 1565

CLXXI. The absorption of water by gelatin. VI. The influence of the thickness and original concentration of the gel. By D. Jordan-Lloyd. (With seven figures) 1580

CLXXII. The effects of cyanide and some other salts on fermentation by yeast preparations. By L. M. B. Patterson. (With three figures) . . 1593

CLXXIII. A spectrographic analysis of human tissues. By J. H. Sheldon and H. Ramage. (With Plate VII) 1608

CLXXIV. Blood-phosphorus in fat absorption. By B. Vahlquist . 1628

CLXXV. The determination of tryptophan and tyrosine. By G. A. da Silva 1634

CLXXVI. Effect of bacteriophage on the oxidation-reduction potentials of *B. coli communis* cultures. By L. F. Hewitt. (With six figures) . . 1641

PAGE

CLXXVII. The influence of sunlight on milk. By H. R. Whitehead. (With two figures) 1647

CLXXVIII. A method for the direct determination of urea in urine. By S. W. Cole 1653

CLXXIX. The accessory factor necessary for the growth of *Nematospora gossypii*. I. The chemical nature of the accessory factor. By H. W. Buston and B. N. Pramanik 1656

CLXXX. The accessory factor necessary for the growth of *Nematospora gossypii*. II. The relation of the accessory factor to "bios." By H. W. Buston and B. N. Pramanik 1671

CLXXXI. The rôle of certain dietary factors in the formation of erythrocytes. By B. C. Guha and L. W. Mapson. (With six figures) 1674

CLXXXII. A survey of anthocyanins. I. By G. M. Robinson and R. Robinson 1687

CLXXXIII. An anthocyanin of *Oxycoccus macrocarpus* Pers. By K. E. Grove and R. Robinson. (With one figure) 1706

CLXXXIV. The ergothioneine content of the blood in health and disease. By H. B. Salt. (With one figure) 1712

CLXXXV. The colorimetric determination of uric acid in urine. By H. B. Salt 1720

CLXXXVI. Blood-phosphatases. By J. Roche. (With four figures) . 1724

CLXXXVII. Effect of addition of salts on the isoelectric point of proteins. II. By S. J. Przyłęcki and W. Giedroyć 1734

CLXXXVIII. Studies in the sulphur metabolism of the dog. IX. The synthesis of phenyl sulphate and indoxyl sulphate. By T. S. Hele. (With three figures) 1736

CLXXXIX. Investigations on the determination of vitamin D. Comparison between the preventive and the curative methods. By P. Schultzer 1745

CXC. The blood-sugar level in vitamin B_1 deficiency. By M. E. Bell. (With one figure) 1755

CXCI. The biological decomposition of plant materials. V. Some factors determining the quantity of nitrogen immobilised during decomposition. By E. H. Richards and A. G. Norman 1769

CXCII. The biological decomposition of plant materials. VI. The effect of hydrogen ion concentration on the rate of immobilisation of nitrogen by straw. By A. G. Norman 1779

CXCIII. Experiments on nutrition. XI. Comparative vitamin B_1 values of foodstuffs. Fruits and vegetables. By R. H. A. Plimmer, W. H. Raymond and J. Lowndes 1788

CXCIV. The significance of the Michaelis constant for saccharase. By G. E. Briggs 1801

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CXCVIII. The micro-determination of sodium in biological materials. By R. A. McCance and H. L. Shipp 1845

No. 6

PAGE

OBITUARY NOTICE 1849

CXCIX. The formation of bacterial proteases, especially in synthetic media. By R. B. HAINES 1851

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CCXIX. The hemicelluloses of the wood of English oak. I. The effect of the drying of wood on the yield and composition of hemicellulose A. By M. H. O'DWYER 2017

PAGE

CCXX. The chemistry of the white rots of wood. II. The effect on wood substance of *Armillaria mellea* (Vahl.) Fr., *Polyporus hispidus* (Bull.) Fr., and *Stereum hirsutum* Fr. By W. G. Campbell 2023

CCXXI. Further observations on the oxidation of lactic acid by brain tissue. By C. A. Ashford and E. G. Holmes. (With seven figures) . . 2028

CCXXII. The distribution of the vitamin B complex. III. Fruits. By M. H. Roscoe 2050

CCXXIII. The effects of coprophagy in rats deprived of the vitamin B complex. By M. H. Roscoe. (With one figure) 2056

CCXXIV. Oxidation-reduction potentials of *Staphylococcus* cultures. II. Effect of bacteriophage. By L. F. Hewitt. (With two figures) . . . 2068

CCXXV. Synthesis and crystal spacings of certain long-chain paraffins, ketones and secondary alcohols. By S. H. Piper, A. C. Chibnall, S. J. Hopkins, A. Pollard, J. A. B. Smith and E. F. Williams. (With Plate VIII and nine figures) 2072

CCXXVI. The wax constituents of the apple cuticle. By A. C. Chibnall, S. H. Piper, A. Pollard, J. A. B. Smith and E. F. Williams 2095

CCXXVII. The wax constituents of forage grasses. I. Cocksfoot and perennial ryegrass. By A. Pollard, A. C. Chibnall and S. H. Piper . . 2111

CCXXVIII. The dicarboxylic acid-nitrogen of proteins. With a note on the alcohol-soluble protein from ragi (*Eleusine coracana*). By M. Damodaran 2123

CCXXIX. Vitamin A and carotene. VIII. High potency vitamin A concentrates. By T. Moore. (With one figure) 2131

CCXXX. A study of the factors influencing the concentration of complement in the blood. By T. W. B. Osborn. (With three figures) . . 2136

CCXXXI. On the function of torulin. An *in vitro* effect of antineuritic vitamin concentrates. By N. Gavrilescu and R. A. Peters. (With one figure) 2150

CCXXXII. The assimilation of aluminium by the human system. By S. J. Lewis 2162

CCXXXIII. Note on the activation of oxido-reductions by co-zymase. By H. v. Euler and R. Nilsson 2168

CCXXXIV. "Resting" bacteria. By B. R. Sandiford and W. R. Wooldridge. (With three figures) 2172

CCXXXV. The nitrogen metabolism of the lupin seedling. By P. McKie. (With one figure) 2181

CCXXXVI. The basic amino-acids of wool. By A. M. Stewart and C. Rimington 2189

I. THE EFFECT OF HEAT UPON THE ACTIVATING EFFICIENCY OF ENTEROKINASE.

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(*Received November 6th, 1930.*)

The effect of heat upon the properties of enterokinase has not been studied in very great detail, and the work that has been done is not of a quantitative nature. Mellanby and Woolley [1913] stated that enterokinase is destroyed in 5 mins. at 65°, and is immediately inactivated in the presence of free acid. In more recent work Waldschmidt-Leitz [1924, 1925] has shown that enterokinase solutions are inactivated when heated above 50° and that they are most stable about the neutrality point. The work described in this paper deals with a quantitative investigation of the effect of heat upon enterokinase. This has been effected in principle by maintaining an enterokinase solution at a temperature of 55°, removing samples at various time intervals, and then measuring the enterokinase content of a definite volume of each sample. The procedure involves two consecutive stages.

(1) Addition of a definite volume of the solution to a constant volume of buffered trypsin solution, leaving the mixed solutions at 30° for 30 mins.[1], when formation of the compound trypsin-kinase takes place.

(2) Addition of caseinogen and buffer solution to the trypsin-kinase at the end of the above period, and then measurement of the amount of hydrolysis of the caseinogen produced in 20 mins., reckoned from the time of addition of caseinogen to the trypsin-kinase.

Before carrying out the above steps however it is necessary to define an arbitrary enterokinase unit.

The determination of enterokinase.

For the study of the effect of heat upon enterokinase it is necessary to have a method for the measurement of "quantity" of enterokinase. This measurement is arrived at by determining the activating capacity of a sample of enterokinase with respect to a known and constant quantity of trypsin. In this way both Waldschmidt-Leitz [1925] and Linderstrøm-Lang and Steenberg [1929] have defined arbitrary enterokinase units. In their determinations

[1] The diminution in the enterokinase content during this period of "activation of the trypsin" is negligible.

however the quantity of enterokinase was defined from measurements of its activating capacity with respect to dried pancreas powder or to a glycerol extract of the powder. In the present work, to avoid any possible complications from the presence of the activator in the dried gland powder, the enterokinase "quantity" has been measured by determining its activating capacity with respect to trypsin which has been purified so as to be free from enterokinase and the enterokinase pre-stage, according to the method of Waldschmidt-Leitz and Linderstrøm-Lang [1927]. Except for this modification the technique adopted for the determination of an enterokinase unit is essentially that of Linderstrøm-Lang and Steenberg [1929].

Preparations.

(*a*) *Enterokinase solution.* Pig's intestinal mucous membrane was treated with acetone, acetone-ether mixture and finally ether according to the procedure of Waldschmidt-Leitz [1924]. 6 g. of the dried mucosa prepared in this way were dispersed through 200 cc. of distilled water by shaking, and the dispersion was left for 24 hours in the ice chest, to allow the active material to pass into pseudo-solution. The suspension was then centrifuged and filtered. To 100 cc. of the clear filtrate were added 1·6 cc. of *N* acetic acid, and the resulting precipitate of protein was filtered off. The clear supernatant liquid containing the enterokinase was neutralised with *N* ammonia and kept in the ice chest. About 300 cc. of solution were prepared in this way and were used throughout the work described in this paper by diluting a volume of 10 cc. with 3 cc. of distilled water.

(*b*) *Trypsin solution.* Glycerol extract of dried pancreas powder was purified according to the method of Waldschmidt-Leitz and Linderstrøm-Lang [1927], so as to be free from enterokinase and the pre-stage of that activator. The details of this purification are given in a previous paper by the present writer [1930].

(*c*) *Caseinogen solution.* The caseinogen solution was prepared by adding 100 cc. of 0·05 *N* ammonia to 6 g. of caseinogen (Kahlbaum-Hammarsten) stirring and keeping for 1 hour at 30°. This solution is referred to as a 6 % caseinogen solution.

(*d*) *Buffer solutions.* Buffer (1) was prepared by mixing 300 cc. of *N* ammonium chloride and 50 cc. of *N* ammonia, giving a p_H of 8·3 at 30°.

Buffer (2) was prepared by mixing 250 cc. of *N* ammonia and 350 cc. of distilled water.

Procedure.

The determination of an enterokinase unit according to the method of Linderstrøm-Lang and Steenberg [1929] was then carried out as follows. 3·5 cc. of buffer (1) and 3 cc. of trypsin solution were measured into a 50 cc. Jena flask. There was then added a volume of distilled water such that after the addition of the required amount of enterokinase solution the total volume

was 9 cc. The flask with its contents was placed in the thermostat at 30° and the enterokinase solution was added at a time which was reckoned as the zero for the period of activation. (5 cc. of water, 65 cc. of 96 % alcohol and 1 cc. of a 1 % solution of thymolphthalein in alcohol were measured out into each of two 300 cc. flasks to be used for the titration of the mixture under investigation before and after the hydrolysis of the caseinogen.)

After the activation period of 30 mins., 6 cc. of buffer (2) (warmed to 30°) and 15 cc. of a 6 % caseinogen solution (also previously warmed to 30°) were added. The whole was shaken and, at a time reckoned as the zero for the period of hydrolysis, 10 cc. of the mixture were pipetted into one of the titration flasks containing alcohol. (The alcohol entirely stops the enzyme action without perceptibly precipitating the caseinogen, thus making it possible to keep the mixture for later titration.) At the end of the reaction period of 20 mins. the same 10 cc. pipette was rinsed with the liquid under investigation, and 10 cc. were pipetted into the second titration flask containing alcohol. Each titration flask contained one-third of the amounts used in the experiment. The contents of each flask were then titrated in the stepwise manner described by Willstätter *et al.* [1926]. The difference between the two titration values is equal to the number of carboxyl groups formed during the hydrolytic period of 20 mins. in 10 cc. of the experimental liquid, expressed as cc. of 0·2*N* NaOH.

By using various quantities of kinase solution and making measurements in this way a relation was obtained between the quantity of enterokinase and the extent of the hydrolysis of the caseinogen. This is shown in Table I.

Table I.

Volume of enterokinase solution cc.	Increase in acidity cc. 0·2*N* NaOH
0·05	0·53
0·10	0·80
0·20	1·04
0·30	1·12
0·50	1·19

An enterokinase unit was defined as the quantity of enterokinase which produces an increase in acidity equivalent to 1·04 cc. of 0·2*N* NaOH under the conditions given above. By plotting the results of Table I and using this definition of an enterokinase unit the following values, set down in Table II, were obtained for fractions and multiples of the unit.

Table II.

Enterokinase units	Corresponding increase in acidity cc. 0·2*N* NaOH
0·25	0·52
0·50	0·80
1·00	1·04
1·50	1·12
2·00	1·18
2·50	1·19

These results are plotted in Fig. 1, and the curve thus obtained is used for determining "quantity" of enterokinase.

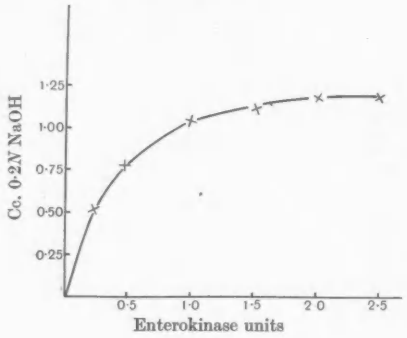

Fig. 1.

The velocity of inactivation of enterokinase by heat.

Having now established a unit of enterokinase activity, we can proceed to measure the rate of thermal inactivation.

To 10 cc. of the enterokinase solution, prepared in the manner described above, were added *N* acetic acid or *N* ammonia to bring it to a required p_H, followed by distilled water to make the total volume 11 cc. This volume of solution was measured into a number of test-tubes already immersed in a thermostat at 55°. The test-tubes were then tightly stoppered and transferred at various time intervals from the thermostat into crushed ice. The enterokinase content of 0·2 cc.[1] of solution was then measured in the manner described above. The p_H measurements throughout the work described in this paper were made at room temperature by means of the glass electrode.

In the following tables the enterokinase content is expressed in terms of the enterokinase unit defined previously and is set down as enterokinase units per cc. of solution. The readings for each time interval were made in duplicate and the enterokinase content recorded for a particular time interval is the mean of the two readings.

The course of the reaction. There is no reference in the literature to the course of the reaction when enterokinase is inactivated by heat. It was found in this work that the effect of heat upon the activating efficiency of enterokinase could be expressed fairly well by the unimolecular expression:

$$k = \frac{2{\cdot}303}{t} \log_{10} \frac{a}{a-x}$$

where a is the initial concentration of enterokinase per cc., $a - x$ is the concentration of enterokinase after time t mins.

[1] This volume was chosen so as to bring the readings on the "steep" part of the curve. If too large a volume is chosen we are in the region of maximum activation and consequently there may be no apparent decrease on heating.

The agreement with the unimolecular expression is illustrated in Table III, which gives some idea of the degree of constancy obtained in average experiments. In some of the experiments there was a tendency for the constants, worked out on the basis of the unimolecular law, to fall. It could not be decided whether this was a real effect or whether it was due solely to experimental error. It should be pointed out in this connection, however, that the experimental observations have not been confined to early stages of the reaction, but that they have covered about 75 % of its course.

Table III.

Temperature 55°.

	Time (mins.)	Enterokinase units per cc.	k*
p_H 8·47	0	6·00	—
	20	3·56	$2·61 \times 10^{-2}$
	40	2·12	$2·59 \times 10^{-2}$
	60	1·37	$2·46 \times 10^{-2}$
p_H 6·40	0	6·00	—
	45	3·50	$1·20 \times 10^{-2}$
	95	2·00	$1·16 \times 10^{-2}$
	143	1·25	$1·10 \times 10^{-2}$

* In this table and throughout k represents the velocity constant of a unimolecular reaction.

The effect of p_H upon the inactivation of enterokinase by heat.

The p_H of the medium has a marked effect upon the stability of enzymes, and the following results, which show that the stability of enterokinase is also dependent upon p_H, emphasise how closely this activator resembles an enzyme in certain of its properties. In Table IV the results obtained for the inactivation of enterokinase by heat at various p_H values are summarised. The mean of the values obtained for k at a particular p_H are set down opposite that p_H value.

Table IV.

Temperature 55°.

p_H	$k \times 10^2$
4·00	3·29
4·56	2·67
5·12	1·30
6·40	1·15
7·83	1·30
8·47	2·55
9·01	6·06

The results of Table IV are presented graphically in Fig. 2, where it is seen that the optimum stability region for enterokinase is fairly broad and lies between p_H 6 and p_H 7.

The critical increment of the process of the inactivation of enterokinase by heat.

Experiments were carried out to determine the critical increment of the heat-inactivation of enterokinase by measuring k at 50° and at 60° for the

following three cases: (1) at the region of optimum stability, *i.e.* at p_H 6·5, (2) at p_H 5·12, (3) at the still more acid reaction of p_H 3·2.

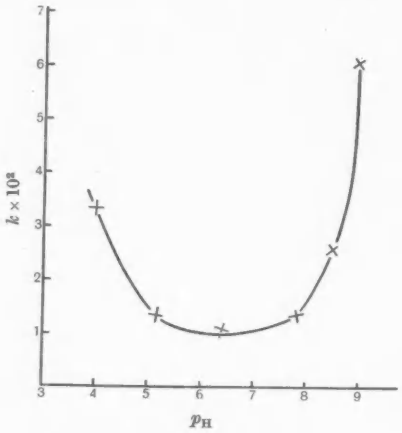

Fig. 2.

Table V.

p_H	k at 50°	k at 60°	E (cals.)
6·50	$4{\cdot}42 \times 10^{-3}$	$3{\cdot}27 \times 10^{-2}$	42,600
5·12	$4{\cdot}95 \times 10^{-3}$	$3{\cdot}52 \times 10^{-2}$	41,700
3·20	$1{\cdot}06 \times 10^{-2}$	$8{\cdot}57 \times 10^{-2}$	44,000

The results are summarised in Table V in which the figures in the last column represent the critical increments calculated by substituting the mean values of k at 50° and 60° into the integrated form of the equation:

$$\frac{d \ln k}{dT} = \frac{E}{RT^2}.$$

Thus it is seen that the critical increment for the heat inactivation of enterokinase is the same over the p_H range 3–7 and is of the order of 40,000 cals. per "molar unit" of enterokinase. This value of the critical increment is substantially the same as that obtained by the writer [1930] for the heat-inactivation of trypsin itself.

It is interesting that the results obtained with enterokinase, a specific activator and not an enzyme *per se*, show such a close resemblance to those obtained with the enzyme trypsin.

Summary.

Experiments have been carried out on the determination of an enterokinase unit. The method adopted was that of Linderstrøm-Lang and Steenberg, except that trypsin purified so as to be free from enterokinase and its pre-stage was used instead of a glycerol extract of the dried pancreas gland.

In this way an enterokinase unit has been defined and employed in the work described later.

The effect of heat upon enterokinase has been studied. It was found that the course of the heat-inactivation may be described fairly well by the unimolecular expression. In this respect enterokinase resembles purified trypsin.

The effect of p_H upon the stability of enterokinase has also been investigated. It was found that the stability is influenced considerably by the p_H of the medium, but there is a fairly broad optimum region of stability lying between p_H 6 and p_H 7.

The critical increment of the process has been determined at three p_H values: 6·5, 5·12 and 3·2. It was found that the critical increment is the same at all three points and is of the order of 40,000 cals. In this respect again enterokinase exhibits a marked resemblance to purified trypsin.

The above work was carried out under the direction of Prof. W. C. M. Lewis, to whom the writer is indebted for help and advice.

The writer desires to express his thanks to the Department of Scientific and Industrial Research for a Maintenance Grant, and also to Imperial Chemical Industries, Ltd., for a grant to the Department of Physical Chemistry of the University of Liverpool.

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II. ON THE QUANTITY OF FREE WATER IN THE RED BLOOD CORPUSCLES.

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(*Received November 24th, 1930.*)

The quantity of free water in the red blood corpuscles is of great significance for a wide field of research, and particularly for the study of the distribution of diffusible substances between the corpuscles and plasma.

In all previous work in this field [Warburg, 1922; Van Slyke, 1926; Henderson, 1928], Ege's [1922, 1] figure of 35–40 % for the disperse phase of the corpuscle, *i.e.* 65–60 % for the free water, has been the basis of calculation. With this value directly or indirectly inserted in the formulae, it has been shown that the distribution of Cl^- and HCO_3^- is inversely proportional to the distribution of H^+. The value of free water only enters the calculation of concentrations of the first named ions, as $[H^+]$ is measured directly as the activity of the blood cell interior. Netter [1929] in the same way has shown that the distribution of NH_4^+ and H^+ are directly proportional. $[H^+]$ here is likewise measured directly, $[NH_4^+]$ computed from the analyses with the value of free water taken as 65 %.

This is all quite in accordance with the Gibbs-Donnan theorem and forms, I think, a strong argument in favour of the above-mentioned value or the free water.

Determination of the quantity of free water in the erythrocytes can be made in several ways, all of them indirect. That most commonly used is the measurement by the hematocrit method of the volumes of the same mass of red corpuscles in several hypo- and hyper-tonic solutions of the same salt. The values found are inserted in the equation for van't Hoff-Boyle-Mariotte's law: $(v - x)\, p = (v_1 - x)\, p_1$, where v and v_1 are the volumes of the cells in solutions of osmotic pressures p and p_1 respectively; the volume of the blood in its own plasma being taken as 100, x then represents the disperse phase and $100 - x$ the free water. Now, Krevisky [1930] has determined this quantity once more and found it to be only 35 %, thereby confirming an older finding by Gough [1924]. Ege [1927] showed that the technique employed by Gough was not irreproachable, giving too high volume values and therefore apparently too small a quantity of free water.

Krevisky uses a high-speed centrifuge until constant volume is reached, but he has only applied highly hypertonic (3–20 %) NaCl solutions as exterior

fluid. In such solutions Koeppe's criterion (*i.e.* a transparency of the column in the hematocrit tube) does not appear and haemolysis very often occurs. The cause of the non-appearance of Koeppe's phenomenon is perhaps that the blood-corpuscles in such solutions assume "thorn-apple" form and therefore cannot pack so closely that all fluid between them is pressed out, this being the condition for the appearance of transparency. The volume values in such concentrated salt solutions are higher than expected according to the above equation, from the values found for v and x in hypo-, iso- and slightly hyper-tonic solutions of the same salt—even if centrifuging is carried on for long periods. Krevisky, in order to make this law valid, enlarges the value of x up to 65 %. This is only what Ege [1922, 2] already observed. The latter author found the following values with dog's blood centrifuged at 8000 r.p.m. for 3–4 hours:

NaCl %	Δ	vol.	x calculated by present author
0·91	0·56	100	—
1·67	1·00	75	43
5·0	2·95	62·4	53·6
6·7	4·95	68·2	64
10·0	5·90	67·4	64

With Ege's volume values in concentrated NaCl solutions inserted in the formula, one finds apparent x values of exactly the same magnitude as Krevisky found in the same solutions. It is not however permissible to assume the extension of the validity of these values to any other range of concentration, be it higher or lower, especially when this has not been investigated. All volume-concentration curves not exceeding about $1\frac{1}{2}$ times the isotonic concentration (Krevisky's lowest is about 3 times isotonic) yield an x value of about the same order of magnitude as Ege's (35–40 %) [*cf.* Christensen and Warburg, 1929].

Whether this discrepancy between the values of x in high and low ranges of concentration is due to a real alteration in the magnitude of x or only to the impossibility of getting correct volume values in concentrated salt solutions by the hematocrit method, I am not at present able to say.

Summary.

The volume values of blood corpuscles in isotonic, hypotonic and slightly hypertonic salt solutions, when inserted in the equation for van't Hoff-Boyle-Mariotte's law, yield values of about 65 % for the quantity of free water. The same blood in highly hypertonic solutions of the same salt gives lower values for the quantity of free water—down to 35 %. The first-named value has been employed in all theoretical work up till now with good result; the value of 35 % must be either fallacious, or only valid in very concentrated solutions.

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III. HYDROXYPYRUVIC ALDEHYDE: ITS PREPARATION AND PHYSIOLOGICAL BEHAVIOUR.

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Preparation of hydroxypyruvic aldehyde.

The general methods for the preparation of α-keto-aldehydes, devised by Wohl and Lange [1908] and Meisenheimer [1912] and developed so successfully by Dakin and Dudley [1914], being inapplicable to the case of hydroxypyruvic aldehyde, it is not surprising that this compound remained unknown until 1926, when its preparation was described by Evans and Waring [1926].

The method adopted by these workers consists essentially in the oxidation of dihydroxyacetone by an excess of finely powdered copper acetate suspended in water. Oxidation is allowed to proceed for 7–10 days at room temperature, when the precipitated cuprous oxide is removed by filtration and the filtrate is freed from the excess of copper by hydrogen sulphide. The resulting "golden yellow" solution is then concentrated under diminished pressure to an oil, which when the pressure is lowered to 9 mm. forms white crystals (M.P. 99°). The yield is 80 % of the theoretical, and the results of qualitative tests and molecular weight determinations by the cryoscopic method, accord with the supposition that the white crystalline material is a trimeride of hydroxypyruvic aldehyde.

Following the details for the oxidation, as described by Evans and Waring, we have confirmed that the weight of cuprous oxide produced after a period of 8 days corresponds with 97·2 % of the theoretical amount required for the usage of one equivalent of oxygen. We doubt, however, whether it is correct to conclude from this result that hydroxypyruvic aldehyde is the sole product of the reaction, and we have failed so far to obtain the crystalline trimeride. Kermack, Lambie and Slater [1929] were also unsuccessful in obtaining a crystalline product under the conditions indicated by the American workers, but claimed that the syrup produced rapidly crystallised if the oxidation had been allowed to continue for 30 days at room temperature. With this modification, we found that the cuprous oxide was contaminated with a greyish white crystalline precipitate of copper oxalate, and, moreover, the total weight of cuprous oxide was then greater (by 19–20 %) than that required for one oxygen equivalent. Hence the resulting product was less pure and was in

fact shown to contain other oxidation products including oxalic acid. The syrupy product when the pressure was diminished to 9 mm. soon passed into a pseudocrystalline mass, which was readily detached from the distilling flask and melted indefinitely just under 100° to a clear yellow liquid. It showed no crystalline structure, and no crystallising medium could be discovered. When the dry material was warmed at 4 mm. pressure, a sublimate of anhydrous oxalic acid formed, and on further heating decomposition of the bulk of the material took place. The product obtained by Evans and Waring's method showed similar behaviour, but yielded no sublimate and was thus probably free from oxalic acid. It may be mentioned that Friedemann [1927] had employed the same method for the preparation of a solution of hydroxypyruvic aldehyde, but made no attempt to isolate the product in the solid state. He considered the oxidation complete after 7 days at room temperature. We have formed the opinion that oxalate formation begins soon after this period, and at any rate is evident after 10 days, when separation of copper oxalate occurs.

The pseudocrystalline solid, obtained as indicated above, was found to be insoluble in all the ordinary solvents except water and alcohol. In the dry state it was practically odourless, but its alcoholic or aqueous solution soon developed an offensive smell characteristic of sulphur compounds, and later sulphur was gradually and continuously deposited. Evans and his co-workers [1928], in their experiments on the oxidation of sugars with copper acetate, add sodium chloride to prevent the formation of colloidal cupric sulphide, but make no mention either of this difficulty or of the presence of sulphur compounds in their preparation of hydroxypyruvic aldehyde. Kermack, Lambie and Slater, however, state that it is difficult to free the product from hydrogen sulphide and colloidal sulphur; in our material the presence of a sulphur derivative was further indicated by the fact that the solutions gave a very marked reaction with sodium nitroprusside. In other respects our product exhibited all the reactions of hydroxypyruvic aldehyde. Both the phenylosazone and the *p*-nitrophenylosazone were obtained in good yield, although during their formation hydrogen sulphide was evolved from the reaction mixtures. The phenylosazone after crystallisation from benzene melted at 132–3° and decomposed about 170°: while the crude *p*-nitrophenylosazone melted with decomposition at 234°, and after recrystallisation from nitrobenzene decomposed sharply at 292°, and had N 23·02 % ($C_{15}H_{14}O_5N_6$ requires N 23·46 %). Evans and Waring quote 230–2° as the melting-point of this compound, but this value is evidently too low, as Dakin and Dudley [1913] describe the compound as decomposing with the evolution of much gas at 315°. Further, it is to be noted that the formation of these osazones is no proof of the presence of the keto-aldehyde in the product, since the same osazones would arise from dihydroxyacetone. However, we found (1) that the product from the oxidation forms the osazones much more readily than does dihydroxyacetone, and (2) that the crop which separates after 4 hours'

interaction between *p*-nitrophenylhydrazine and dihydroxyacetone has a lower melting-point (243°) than the second crop separating after 36 hours (257°). This is probably due to the presence of varying amounts of hydrazone along with the osazone, as the crop melting at 243° had N 21·04 %, a value intermediate between those of the hydrazone (18·67 %) and osazone (23·46 %).

As a freshly prepared aqueous solution of the pseudocrystalline material gave no immediate Schiff reaction, whereas a solution previously boiled did so at once, the product was evidently present in a polymerised form as suggested by Evans and Waring. The molecular weight determinations quoted by these workers, however, are rendered somewhat unreliable owing to the presence of by-products containing sulphur. The exact nature of these by-products has not been determined so far, but it may be recalled that aldehydes in acid solution on treatment with hydrogen sulphide give rise to complexes containing the corresponding thioaldehydes, which on oxidation yield sulphones, a class of compounds with well recognised hypnotic properties. Accordingly, compounds of this character may here be involved, and we have invariably found it impossible, with the hydrogen sulphide method, to obtain a product, which gives no nitroprusside reaction. The main product of the action of copper acetate on dihydroxyacetone is probably hydroxypyruvic aldehyde, but while the excess of copper is most conveniently removed by means of hydrogen sulphide, the use of the latter introduces a complication.

Being unsuccessful with Evans and Waring's method and being in need of a reliably pure specimen of hydroxypyruvic aldehyde in order to test its physiological action, we have tried other methods of preparation. Thus the phenylosazone was prepared from dihydroxyacetone and experiments were carried out to transform it into the osone (1) by Fischer's method with fuming hydrochloric acid, and (2) by the benzaldehyde method. Unfortunately neither process proved successful; in the former extensive decomposition occurred, and the osazone proved too insoluble in water for the latter method to be applicable.

An experiment in which copper acetate was replaced by the corresponding silver salt led to a curious result. After allowing the oxidation to proceed, and subsequently removing the excess of silver by addition of dilute hydrochloric acid, there was obtained on evaporating the solution *in vacuo* a white amorphous product, resembling starch in appearance. It was free from inorganic material, but had no definite melting-point. Once isolated it was sparingly soluble in all solvents, but an aqueous suspension of the material reduced Fehling's solution vigorously in the cold. The sparing solubility of the product precluded the testing of its physiological action, but it was considered to be either a highly polymerised form of hydroxypyruvic aldehyde, or a condensation product analogous to that described by Levene and Walti [1928]. The material has so far not been further examined, but, as a second experiment yielded a somewhat similar result, its preparation is being repeated on a larger scale.

Attempts were then made to modify the copper acetate method of Evans and Waring, avoiding the use of hydrogen sulphide. The oxidation was carried out exactly as described by the American workers, and various methods were then tried for the removal of the excess of copper from the green-coloured filtrate. The only process which proved successful was the careful addition, with cooling and stirring, of saturated baryta until the acid reaction to litmus disappeared. The bulky precipitate containing the cupric hydroxide settled on standing overnight, and the colourless filtrate was freed from barium by means of sulphuric acid, excess of acid being carefully avoided. The filtered solution was then concentrated *in vacuo* to a syrup. This was extracted with alcohol and the filtered alcoholic extract was again evaporated under diminished pressure to a syrup, which under 6–9 mm. passed into the pseudo-crystalline state. The product obtained in this way had all the properties of a polymeride of hydroxypyruvic aldehyde. An unheated solution gave no immediate reaction with Schiff's reagent, but a solution which had been heated for a few minutes and subsequently cooled gave a marked reaction at once: it also reduced Fehling's solution in the cold and gave an intense coloration with phosphotungstate, but a negative test with sodium nitroprusside. It gave a purplish-red coloration with the phenol reagent used by Kermack, Lambie and Slater [1926] for the detection and estimation of dihydroxyacetone, and readily formed a *p*-nitrophenylosazone, M.P. 285°. No attempt was made to estimate by molecular weight determinations the degree of polymerisation, as this will probably vary in different experiments. The material was not definitely crystalline, but was undoubtedly hydroxypyruvic aldehyde, and not being contaminated with sulphur derivatives was purer than that previously described. In view of the ease with which keto-aldehydes are affected by alkali, a control experiment was carried out, in which a mixture of glyoxal and copper acetate was subjected to the same baryta treatment as outlined above. The glyoxal was quite unaffected by the process.

Physiological action of hydroxypyruvic aldehyde.

As already reported [Hynd, 1930], the toxic action on mice of hydroxypyruvic aldehyde prepared by the method of Evans and Waring was observed by me 3 years ago, but the matter was not further investigated until Kermack, Lambie and Slater [1929] published their paper claiming that the dimeric form of hydroxypyruvic aldehyde produced symptoms similar to those following the administration of glucosone.

The material obtained by the methods described above has been injected subcutaneously into normal mice, and the effects produced have been carefully studied in a large number of experiments. Some typical cases are detailed in Table I, in which are also included experiments in which charcoal was employed to remove colloidal copper sulphide from the filtrate after the treatment with hydrogen sulphide. As we were concerned mainly with the effect of hydroxypyruvic aldehyde prepared by different processes, the con-

centration of the solutions used for injection was in all cases 15 %, and a uniform dose of 0·05 g. per 20 g. mouse was employed.

Table I. *Effect of subcutaneous injections into normal mice of hydroxypyruvic aldehyde prepared by different methods.*

Method of preparation	Test with nitro-prusside	Behaviour of animal	General inference
I. Copper removed by hydrogen sulphide	+	1. Became inactive almost at once. After 1 minute legs sprawling: then began to gasp. Rolled over on its side, and lay comatose. Two minutes after injection, animal suddenly righted itself: then followed general convulsive movements. Dead in 3 minutes after injection	The material has a very rapid effect, and is highly toxic
Do.	+	2. Breathing difficult after 1 minute. After 2 minutes convulsion, followed by slight recovery, when animal walked about in an excited condition. A second convulsion after 4 minutes, then coma and death in 6 minutes	Do.
Do.	+	3. After 2 minutes very irritable, sprawling limbs, rapid breathing. Jumps if handled. Convulsion in 4 minutes: dead 5 minutes after injection	Do.
II. Copper removed by hydrogen sulphide and the filtrate shaken with charcoal	+	1. Slight sprawling in 2 minutes. Rapid respiration. Disinclined to walk unless disturbed. Then became very drowsy and remained in that state all day. Found dead next morning	Effect is slow: drowsiness the main symptom
Do.	+	2. Tail erect: very irritable for 5 to 10 minutes after injection. Then gradually became lethargic, and died in 6½ hours	Do.
Do.	+	3. Active for 2 minutes after injection. Then sat quietly. Irritable when disturbed. Very drowsy after 20 minutes. Slight convulsion after 1 hour. Then moribund: died after 3 hours	Do.
III. Copper removed by means of baryta	–	1. No effect was noted during first hour after injection. Then gradually became drowsy and remained so all day. Refused to take food. Normal next day	Slow effect. Drowsiness the main symptom
Do.	–	2. No symptoms except drowsiness, which developed gradually during forenoon. Continued very drowsy all day. Found moribund next morning. Died 26 hours after injection	Do.
Do.	–	3. Slight sprawling 2 minutes after injection. Rapid respirations. Then became drowsy. When disturbed moved about with difficulty. Remained in this condition all day. Normal next morning	Do.

In the above experiments, apart from the concentration of the solution at 30° under diminished pressure, no heating was employed in the course of the preparation of the material used for the injections. All the solutions used gave negative tests to Schiff's reagent. Boiling for 5 to 10 minutes gave solutions, which when cold responded to Schiff's test readily, and the effects of the injection of these solutions are shown in Table II.

Table II. *Effect of subcutaneous injections into normal mice of monomeric hydroxypyruvic aldehyde obtained by different methods.*

Method of preparation	Test with nitro-prusside	Behaviour of animal	General inference
I. Copper removed by hydrogen sulphide	+	1. Walking about 2 minutes after injection. Sprawling after ¼ hour. Then very drowsy and remained so until death in 5¼ hours	Main symptom is drowsiness
Do.	+	2. No definite symptoms developed. Animal remained quiet and drowsy all day. Found dead next morning	Do.
II. Copper removed by baryta	–	1. Slight sprawling after ½ hour. Then very drowsy: but jumps when handled. Found dead next morning	Drowsiness the main symptom
Do.	–	2. Very little effect: tail stiff: then slight sprawling after ½ hour. Drowsy but irritable if disturbed. Normal next morning	Do.

It should also be noted that 30 animals have been injected with hydroxypyruvic aldehyde, prepared by a method involving the use of hydrogen sulphide, and death, within 12 hours, has resulted in every case. On the other hand, of the 15 mice injected with hydroxypyruvic aldehyde prepared by the "baryta" method, 10 recovered completely, and the remainder died after periods ranging from 6 to 48 hours.

Discussion.

Kermack, Lambie and Slater [1929] reported that the monomeric form of hydroxypyruvic aldehyde, although holding the aldehyde group in a more reactive condition, was much less toxic than the "dimeric" form, but furnished no explanation of this somewhat unexpected finding. From the data tabulated above, our results are seen to be in agreement with their observation that the monomeric form of hydroxypyruvic aldehyde does not produce a train of symptoms suggestive of insulin hypoglycaemia. It is also clear that the hydroxypyruvic aldehyde prepared in absence of hydrogen sulphide shows little if any difference when used in the monomeric or polymeric state. Similarly it was found that both monomeric and polymeric glyoxal exert practically the same effect when injected subcutaneously into mice. Moreover, the symptoms produced by glyoxal resemble in every detail those described for sulphur-free hydroxypyruvic aldehyde. Thus, even when a lethal dose is

administered, the onset of symptoms is not rapid: but the animal gradually becomes inactive and drowsy, and when disturbed moves about with difficulty owing to weakness of the limbs. Breathing gradually becomes more shallow and death follows, without convulsions, in 2–6 hours. This is typical of five experiments we have carried out using glyoxal purchased from the British Drug Houses.

We conclude therefore that the very rapid and highly toxic effect observed by Kermack, Lambie and Slater, which we also find to ensue as the result of injecting polymeric hydroxypyruvic aldehyde prepared by Evans and Waring's method, is not due to a difference in molecular complexity of the material used, but rather to the presence of an unstable by-product. This is supported by the fact that treatment with charcoal reduces the toxicity of the product in the same way as heating the solution. Yet, it is to be noticed, the charcoal-treated material gives no Schiff reaction, and hence contains the hydroxypyruvic aldehyde in a polymeric form.

As already indicated, we have failed so far to elucidate the precise nature of the by-product. The impurity which gives rise to the reaction with sodium nitroprusside is not alone responsible for the rapid toxic effect, as this reaction is still marked after charcoal treatment and also after heating. Yet the toxicity is greatly diminished by these procedures. Neither is the toxicity to be explained by the presence of oxalic acid, as we have shown by control experiments that rats withstand much larger doses of this acid than could possibly have been present in the solution of hydroxypyruvic aldehyde injected. Moreover, heating the solution would not materially influence the effect of an injection of oxalic acid.

Whatever the toxic material may prove to be, it is certainly highly unstable. The symptoms which follow on a subcutaneous injection are quite different from those observed after an injection of glucosone and are more suggestive of the effects produced by hydrogen sulphide or colloidal sulphur. Kermack, Lambie and Slater admit that in the preparation of hydroxypyruvic aldehyde it is very difficult to eliminate the last traces of hydrogen sulphide and colloidal sulphur, but conclude that hydrogen sulphide plays no part in the production of the toxic symptoms. From the results of several control experiments with various solutions containing hydrogen sulphide, we are quite satisfied that the amount of preformed hydrogen sulphide in the unheated solution of hydroxypyruvic aldehyde injected would give rise to no toxic symptoms. It is to be remembered, however, that an aqueous solution of the material at laboratory temperature continuously evolves hydrogen sulphide or some similar sulphur-containing product, and at the same time sulphur is deposited. It is conceivable that this process may be considerably accelerated by contact at 37° with the body tissues and fluids.

Again, although the material (*i.e.* sulphur) precipitated on heating the solution had, as shown by Kermack and his co-workers, no toxic effects, it does not follow that the substance from which this precipitate was produced

by heat had no toxic effect. Thus it is usually accepted that the toxicity of sulphur is due to its reduction in the organism to hydrogen sulphide, and that the extent to which the reduction occurs, and hence the toxicity, depends on the physical condition, being most intense in colloidal, less in amorphous, and least in crystalline sulphur. The degree of dispersion is thus an important factor in the effect of a preparation of colloidal sulphur [Messini, 1928]. Further, as is well known, the injection of colloidal sulphur gives rise to a hypoglycaemia and hence to symptoms resembling those following the administration of insulin. It has been pointed out, however, that there is a marked difference between the action of insulin and that of colloidal sulphur, for in the latter case there is no strict proportionality between the amount of colloidal sulphur injected and the intensity of the resulting hypoglycaemia [Pennetti, 1928]. Moreover, the hypoglycaemia is more prolonged and is not relieved by glucose, as it is accompanied by a toxic effect comparable with that caused by asphyxiating substances, such as hydrogen sulphide, or cyanides, on the respiratory centre.

While it seems improbable that colloidal sulphur would withstand the process of drying under a pressure of 9 mm. employed in the preparation of hydroxypyruvic aldehyde, a sulphur-containing by-product having an action similar to that indicated above for colloidal sulphur might conceivably still be present. This view seems to fit in best with the results of further experiments which we have carried out. Thus we have found that similar toxic compounds arise when solutions of dihydroxyacetone and glyoxal are subjected to the action of hydrogen sulphide, but apparently no such compounds form with either the sugars, glucose and fructose, or with glucosone.

The toxic product obtained from glyoxal and hydrogen sulphide differs from that encountered in the preparation of hydroxypyruvic aldehyde in that it is more stable to heat. Boiling a solution of glyoxal, which has been subjected to the action of hydrogen sulphide, gives rise to no turbidity, and death with convulsions results in 3–8 minutes after the injection of an amount of the heated solution containing the equivalent of a dose of glyoxal, which causes death in 2–6 hours. The same dose of the unheated solution of glyoxal treated with hydrogen sulphide causes death in 2–5 minutes.

As the sugars do not give rise to toxic products with hydrogen sulphide, the positive result with dihydroxyacetone may be due to the presence of traces of pyruvic aldehyde in the sample used. The negative result with glucosone is surprising, but it may be that the unchanged glucosone exerts a protective action against the toxic product, just as it antagonises the action of cyanides [Hynd, 1927], which it may be added, give rise to symptoms remarkably similar to those ascribed by Kermack, Lambie and Slater to the "dimeric" form of hydroxypyruvic aldehyde.

The contention of the above-named authors that hydroxypyruvic aldehyde produces symptoms similar to those caused by glucosone is absolutely refuted by the results recorded in the present communication.

SUMMARY.

1. When hydrogen sulphide is used in the preparation of hydroxypyruvic aldehyde, the resulting material is contaminated with sulphur-containing by-products. Some of these are stable to heat, others unstable, and the latter are highly poisonous, producing symptoms similar to those following the administration of cyanides.

2. Somewhat similar toxic compounds arise when dihydroxyacetone and glyoxal are treated with hydrogen sulphide, but apparently not when the sugars and glucosone are so treated.

3. A modification of the method of preparing hydroxypyruvic aldehyde is described whereby a product is obtained free from sulphur-containing compounds.

4. It is also clearly shown that no material difference exists between the effects produced by monomeric and polymeric hydroxypyruvic aldehyde, when injected subcutaneously into mice. The effect of either is practically identical with that which ensues after the injection of glyoxal, the main symptom being drowsiness.

5. The presence of an unstable, highly toxic by-product, which arises through the use of hydrogen sulphide, adequately explains the reported greater toxicity of the "dimeric" form of hydroxypyruvic aldehyde.

6. The glucosone effect is quite distinct from that produced by any of the compounds dealt with in this paper.

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IV. SPECTOGRAPHIC DATA CONCERNING VITAMIN A AND LIVER OILS.

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The object of the present work is to place on record a number of spectroscopic observations on liver oils and concentrates. The data are in the first place concerned with the more precise description of tests for vitamin A, in particular with the ultra-violet absorption and the blue colour test. In the second place, we have utilised spectrographic methods for a more penetrating investigation of the wider problem of the constituents of liver oils and concentrates. We hoped in this way to gain some insight into the sequence of changes culminating in the synthesis of fat-soluble vitamins or provitamins in Nature. Although it is too early to estimate to what extent this hope has been realised, a completely fresh aspect of the chemistry of vitamin-bearing oils emerges from the discovery of new and highly characteristic absorption spectra, which cannot be identified with the properties of the major constituents of liver oils.

It has already been shown [Morton and Heilbron, 1928; Drummond and Morton, 1929] that vitamin A is characterised by a broad, intense absorption band with a maximum near 328 $\mu\mu$. Further experience on a wide range of liver oils and concentrates serves to confirm this view, whilst independent support is given in the work of Moore [1930], Capper [1930] and Drummond, Ahmad and Morton [1930] on the conversion of carotene into vitamin A *in vivo*.

The number of samples of crude and refined oils and of concentrates which have been examined in these laboratories is now considerable, and although the correlation between the ultra-violet absorption, the antimony trichloride colour test and the vitamin A potency is more firmly established than ever, attention must be drawn to the fact that disturbing factors can modify the results to some extent. Liver oils vary very widely and the spectroscopic tests are not immune from interference. Due allowance must therefore be made for disturbances caused by unusual composition of certain oils.

In collating the data on cod-liver oils the following facts emerge.

Liver oils. (1) The ultra-violet absorption of oils either in thin films or in alcoholic solution is characterised by a single well-defined maximum at 320–330 $\mu\mu$. There is some evidence of a slight step-out in the curve near 300–310 $\mu\mu$ and in a few oils of very low vitamin A potency narrow bands

between 300 and 350$\mu\mu$ are faintly shown. A careful examination of the whole of the data, however, satisfies us that the true vitamin A ultra-violet absorption band is in a high degree continuous and free from fine structure.

The vitamin A potency as measured by absorption varies over a somewhat wide range. For a 1 cm. layer of a 1 % solution in alcohol E ($\log I_0/I$, where I_0 = intensity of incident light, I = intensity of emergent light) varies for "genuine refined" cod-liver oils from 0·2 to 2·0. The majority of the oils lie within the limits 0·5 to 1·6. (Carr and Price units are approx. 12 times the above.)

A small fraction of the observed absorption at 328$\mu\mu$ is not due to vitamin A, but to other constituents of the oils. In order therefore for the spectrographic method to be used as a quantitative measure of vitamin A potency, a correction must be applied. For pure weakly coloured cod-liver oils it is generally satisfactory to subtract 0·2–0·3, whilst highly coloured oils of the cattle feeding variety require a somewhat larger correction.

(2) The persistence (*i.e.* $E_{max} - E_{min}$) of the ultra-violet band is variable. This undoubtedly arises from the fact that some fairly absorbent substance other than vitamin A may be present in genuine cod-liver oils, the proportion being liable to considerable variations. The ultra-violet absorption spectrum of any liver oil can therefore be regarded as fixing an upper limit to its vitamin A potency.

(3) Nearly every cod-liver oil exhibits selective absorption in the region 260–295$\mu\mu$. Occasionally it has been possible to record some degree of resolution in this region but for the vast majority of cases only an inflexion near 280$\mu\mu$ can be obtained. No clear parallelism between the absorption at 280$\mu\mu$ and vitamin D potency has been traced, nor has it been possible to associate the absorption with the ergosterol content.

(4) With a few samples of fish-liver oils other than cod-liver oil the absorption at 328$\mu\mu$ is very high but the band appears only as an inflexion. The band is partially masked by an intense abnormal ultra-violet absorption of unknown origin. If however the vitamin A is destroyed by irradiation or aeration and the absorption spectrum is redetermined it is found that the new curve is consistent with the removal of a substance exhibiting the normal vitamin A band.

(5) Spectroscopic examination of the blue solutions with antimony trichloride discloses a maximum which is normally within the limits 604–608$\mu\mu$. Those samples of cod-liver oil which show the 328$\mu\mu$ ultra-violet band clearly and with high persistence also give a blue colour characterised by a single sharp band at 604–608$\mu\mu$ and little or no selective absorption elsewhere in the visible. This class comprises the majority of pale medicinal cod-liver oils and the parallelism between blue colour and ultra-violet absorption is well marked without the introduction of correction factors. With crude cod-liver oils of high potency it is frequently found that the blue solutions exhibit additional selective absorption in the region 565–585$\mu\mu$. Normally this band

appears as a secondary maximum of smaller intensity than the band at 604–608 $\mu\mu$. In one or two samples of cod-liver oil and in a few samples of shark-liver oil the band in the region 565–585 $\mu\mu$ definitely predominates over the normal band. Even in such cases however the 328 $\mu\mu$ band is shown clearly with alcoholic solutions of the original substance; its persistence is unusually low, indicating the presence of additional ultra-violet absorption possibly related to the abnormal colour reaction referred to above.

Further work will be necessary before a decision can be arrived at as to whether the abnormal results are due to a mere displacement of the normal band in the colour test owing to some unusual property of the oil (*e.g.* high content of unsaturated free fatty acid) or to some totally new constituent.

Concentrates. When liver oil concentrates are examined, the blue solutions are found to be characterised by a sharp band with its maximum at 620–624 $\mu\mu$, the intensity varying directly with the intensity of the ultra-violet band at 328 $\mu\mu$ in the original material. The blue solutions with many concentrates also show a less intense band with a maximum at 582–593 $\mu\mu$; when the quantity of concentrate used is sufficient to give very deep blue colours, additional maxima have been seen at 643 and 697 $\mu\mu$ with two separate samples. In the two richest concentrates (from different oils) which we have as yet studied (Carr and Price blue values 14,000 and 9,400 respectively) both the broad continuous ultra-violet band with maximum at 328 $\mu\mu$ and the 624 $\mu\mu$ blue band were clearly exhibited at roughly 1200 and 800 times the intensity shown by a typical cod-liver oil. It is noteworthy that with these concentrates no other maxima were shown.

If concentrates rich in vitamin A are diluted with an inactive oil (*e.g.* seal oil) the quantities being so adjusted that the potency of the product is of the same order as that of cod-liver oil, the absorption maximum in the colour test reverts from 620–624 $\mu\mu$ to the normal position for a cod-liver oil, namely 604–608 $\mu\mu$.

As a result of various chemical treatments which cause decomposition of the material responsible for the characteristic ultra-violet band (328 $\mu\mu$) shown by fish-liver oil concentrates, we have repeatedly observed absorption maxima near 394, 370, 350 and 334 $\mu\mu$. Concomitantly, the concentrates cease to exhibit the 620–624 $\mu\mu$ band with antimony trichloride. The capacity to give a blue colour has not however entirely disappeared for the relatively weakly coloured solutions show a band at 583 $\mu\mu$. Similarly, when a sample of unsaponifiable matter from sheep-liver fat was left in contact with the air for some weeks and extracted with a small quantity of heptane, the extract no longer showed the 328 $\mu\mu$ band but exhibited the same maxima at 394, 369, 349 and 334 $\mu\mu$ (Fig. 1) as were shown by the treated fish-liver oil concentrates. On removal of the heptane and addition of the antimony trichloride reagent a blue solution exhibiting a single band at 583 $\mu\mu$ was again obtained.

At the time that the above observations were made, it was already clear from the work of Drummond and Baker [1929] and from our own experience

that there was little prospect of the direct isolation of vitamin A in a state of purity from liver oils. It was also becoming evident that while the hydrocarbon carotene was not itself vitamin A [Dulière, Morton and Drummond, 1929] it was enormously potent in promoting growth [Euler, Euler and Hellström, 1928; Moore, 1929; Collison, Hume, Smedley-MacLean and Smith, 1929]. The direct attack on vitamin A was in a position of stalemate and the indirect attack through carotene seemed promising. The new absorption bands referred to above seemed worth further study since they might throw light either on the substance from which vitamin A is made or on substances derived from the breakdown of the vitamin molecule.

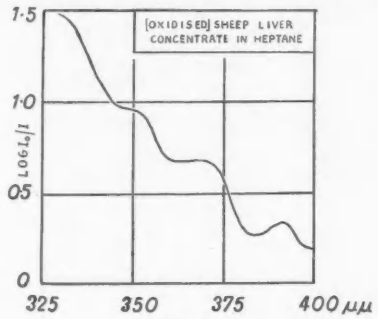

Fig. 1.

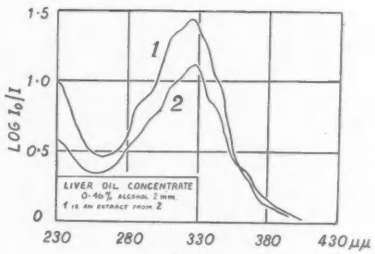

Fig. 2.

We chose first to investigate the decomposition products of the vitamin. Before however reporting on the absorption spectra of concentrates from which the typical ultra-violet band had been removed, it is necessary to reconsider this band. So far, it has been described as a broad continuous band having no recognisable fine structure.

Using a concentrate 200 times as potent as cod-liver oil we obtained indications of fine structure (Fig. 2). Attempts to increase the degree of resolution either by replacing alcohol with hexane as the solvent or by the use of still richer concentrates proved fruitless. As a result of experiments involving distribution between partially miscible solvents a very considerable increase in potency was obtained in one fraction but the indications of fine structure remained quite indefinite. As it was further found that the degree of resolution was less rather than greater, when a concentrate 1000 times as rich as cod-liver oil became available (Fig. 3) it was resolved to proceed on the two assumptions that the vitamin A band was really highly symmetrical and that the indications of fine structure arose from other substances, the relationship of which to vitamin A was unknown.

On this basis the next step was clearly to effect the removal of the 328 $\mu\mu$ band by decomposition of the vitamin in order to see if any fine-banded absorption could be revealed after eliminating the obscuring effect of the main broad band. Numerous trial experiments were made, but only one

need now be mentioned, namely the evaporation of the concentrate with strong alcoholic sodium ethoxide. An extract of this product disclosed on spectrographic examination (*a*) the absence of the 328 $\mu\mu$ band, (*b*) a diminution in intensity of absorption and (*c*) an increase in selectivity. With antimony trichloride a comparatively feeble blue colour was given, but the 624 $\mu\mu$ band was absent, the only absorption band present having its maximum at 583 $\mu\mu$.

As this experiment had proved fruitful, the work was repeated on a larger scale. For this experiment a neutral cod-liver oil concentrate (obtained from Messrs Lever Bros., Ltd.) having a vitamin potency of 2400 Carr and Price units was employed. As a preliminary measure, the saponification equivalent of the concentrate was determined, using a large excess of reagent and applying heat for a longer period than usual. The material was found to be practically

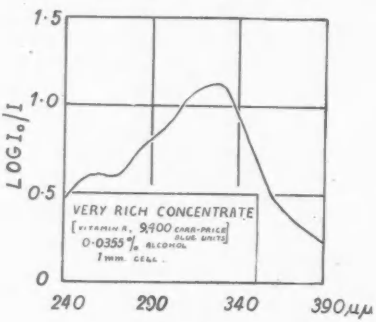

Fig. 3.

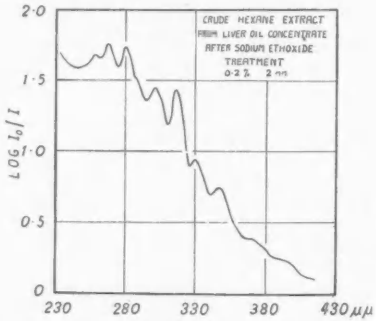

Fig. 4.

free from saponifiable matter (saponification equivalent, 19,000). A further portion of this concentrate was treated as follows. Sodium (25 g.) was dissolved in alcohol (250 cc.) and the solution was heated in an atmosphere of nitrogen in an oil-bath for 2 hours at 150–160° with the concentrate (25 g.). The alcohol was removed under reduced pressure, the residue was dissolved in water (500 cc.) and the whole was twice extracted with ether. The ethereal solution was thoroughly washed with water, dried over sodium sulphate, and evaporated, yielding a dark, brown oil.

The colour test with antimony trichloride, however, revealed the presence of unchanged vitamin A and in consequence the above treatment was repeated. The neutral portion so obtained disclosed (as shown in Fig. 4) on spectrographic examination a series of definite maxima near 394, 375, 350, 330, 316, 302 (inflexion 290), 282, 271 and 260 $\mu\mu$. With antimony trichloride only a band at 583 $\mu\mu$ could be recorded. The combined alkaline liquid from the above experiments was acidified with dilute sulphuric acid and the precipitated oil was extracted with ether. The ethereal solution was shaken with dilute sodium carbonate solution to remove acids, leaving in the ether some neutral resinous material. The acids were precipitated from the sodium carbonate solution and obtained as a dark brown oil (about 2 g.). It will be noted

that the weight of acids isolated is between 5 and 6 times in excess of that demanded by the saponification equivalent (assuming saponifiable matter to be cod-liver oil glycerides) and corresponds to approximately 0·06 % of the original cod-liver oil.

The absorption spectrum of these acids is reproduced in Fig. 5; it will be

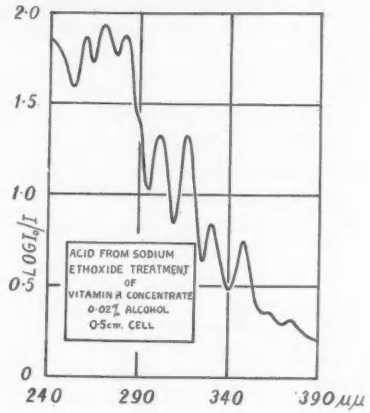

Fig. 5.

observed that this resembles the curve shown in Fig. 4 from which it appears that the characteristic selective absorption is due to acid material. The fact that this is shown in the original so-called neutral portion is due to the solubility of the sodium salts in ether, for by repeated washing of the ethereal solution, first with dilute alkali and then with water, a true neutral material was ultimately obtained in which the fine absorption bands had almost entirely disappeared; with antimony trichloride a violet-blue solution was obtained exhibiting a maximum of considerable intensity at 583 $\mu\mu$.

Owing to the difficulty experienced in accomplishing complete decomposition of the vitamin in the above experiment, it was decided to work at a somewhat higher temperature and for this the alcoholic sodium ethoxide was replaced by sodium amyloxide. The material employed in this case was a cod-liver oil concentrate obtained from Messrs J. Nathan and Co., Ltd. The vitamin A potency was of the same order as that of the Lever extract, but the saponification equivalent in this case was 3900. The material was accordingly re-saponified with 5 % alcoholic potash; the product obtained had then a saponification equivalent of 8200, and was treated with sodium amyloxide in the following manner.

Sodium (12·5 g.) was dissolved in distilled amyl alcohol (125 g.), contained in a 1000 cc. round-bottomed flask fitted with a reflux condenser, and the solution was heated in an oil-bath at 180° in a current of nitrogen. The vitamin concentrate (25 g.) was added, and the oil-bath was kept at 180° for 2 hours,

after which the amyl alcohol was removed as far as possible under reduced pressure. The solid residue was then cooled in an atmosphere of nitrogen and heated with distilled water (250 cc.) until the sodium amyloxide had been decomposed. The product was extracted with ether (1000 cc.), acetone being added to break the emulsions formed. The ethereal extract was repeatedly washed with water, and the washings were combined with the first aqueous extract. This was again extracted with ether until the soaps were entirely freed from neutral material. The alkaline solution was acidified with dilute sulphuric acid, saturated with salt and extracted with ether. The extract was dried over anhydrous sodium sulphate, giving on evaporation the acid portion (3 g.) again in amount considerably in excess of that calculated (0·9 g.) from the saponification equivalent taken as cod-liver oil glycerides. The spectrographic examination of the acid material gave the curve shown in Fig. 6,

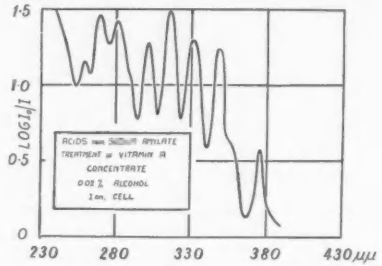

Fig. 6.

revealing the same series of bands as were obtained from the sodium ethoxide treatment. The intensity distribution differed in the two cases and comparison of the respective curves makes it highly probable that the acidic material is heterogeneous, consisting of at least three different selectively absorbing acids. The group of bands at 260, 271 and 282 $\mu\mu$ appears to be due to one substance, the bands at 301 and 317 $\mu\mu$ to another, those on the long wave side of 317 $\mu\mu$ to at least one other acid, selective absorption beyond 260 $\mu\mu$ remaining to be accounted for.

The production of these strongly absorbing acids next led us to examine spectrographically the total acids obtained by the ordinary saponification of cod-liver oil. We found here that the same type of absorption was shown by these acids [for curves see Gillam *et al.* 1931, pp. 34, 35], the ten characteristic bands being equally well-defined. At first sight it would thus appear plausible to suggest that the selective absorption finds its origin in the ordinary fatty acids; this view is however untenable for it fails to account for the absence of this complex absorption from cod-liver oil itself. Calculation of the intensity of the vitamin A band as compared with that of the acids rules out completely the suggestion that the acid bands are masked by the 328 $\mu\mu$ band. Taking the intensity of absorption of a 1 % solution of cod-liver oil using a 1 cm. layer as unity, the intensity of absorption shown by the acids is about 50 times

as great. Masking being therefore eliminated an alternative hypothesis must be sought. Attention may be drawn to two significant points. In the first place it is highly improbable that the usual process of saponification would fail to hydrolyse any materials present in the fat as ordinary glycerides, and in the second place the amount of acidic material obtained from the concentrates by treatment with sodium ethoxide or amyloxide is largely in excess of that calculated from the saponification value.

The highly absorbing acids are therefore unlikely to be simple fatty acids and probably result from drastic and irreversible changes. The richest concentrates of vitamin A after treatment with sodium amyloxide did not exhibit the ten narrow bands, the decomposition products showing only one maximum at 276$\mu\mu$ (Fig. 7). The acids cannot possibly be accounted for as arising solely

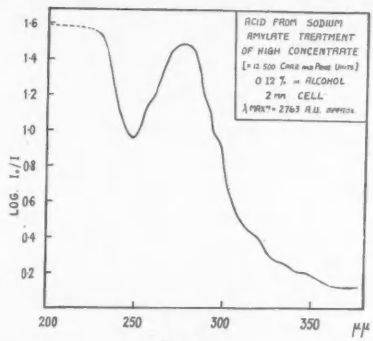

Fig. 7.

from the decomposition of vitamin A because the intensity of the new system of bands is no greater in the preparations from concentrates than in the total acids resulting from the ordinary saponification process, *i.e.* the accumulation of the vitamin in the concentrate is not accompanied by a corresponding increase in the amount of the highly absorbing acids. This experiment seems definitely to eliminate vitamin A itself as a sole contributory factor in the formation of the highly absorbing acids. The retention, in all but the highest concentrates, of material capable of yielding the acids possibly indicates that the bands are associated with either the later stages in the synthesis of vitamin A or the earlier stages of its utilisation in the organism. The band at 276$\mu\mu$ may however well arise as a direct decomposition product of the vitamin, a suggestion supported by the appearance of such a band as a result of the photochemical destruction of vitamin A [Morton and Heilbron, 1928]. Control experiments on the action of sodium amyloxide on both ergosterol and dehydroergosterol have been carried out, and show that decomposition of these substances does not in any way account for the appearance of the absorbing acids.

We are therefore left to conclude that cod-liver oils contain appreciable

quantities of highly transparent substances which are decomposed during the process of saponification giving acids characterised by intense selective absorption. A much smaller quantity of not dissimilar, relatively diactinic material remains undecomposed and passes into the unsaponifiable extract. Drastic treatment with alkali is necessary before this residual, possibly more resistant, material is attacked, but when, as with sodium ethoxide or amyloxide, this occurs the acids produced are of the same type as before.

It is impossible not to be struck by a qualitative similarity between some of the groups of bands and the spectra of ergosterol and dehydroergosterol [Heilbron, Johnstone and Spring, 1929], and the fact that carotene shows a similar set of bands displaced into the visible [Dulière, Morton and Drummond, 1929]. The reappearance of well-resolved bands, again in groups of three, may well indicate that the acids are not unrelated to substances either of the poly-ene or sterol type.

Summary.

1. Precise spectroscopic data regarding the vitamin A ultra-violet absorption band are recorded.

2. The vitamin A band at 328$\mu\mu$ is found to be free from fine structure.

3. Nearly all cod-liver oils exhibit selective absorption in the region 260–295$\mu\mu$.

4. Spectroscopic examination of the blue solutions obtained with antimony trichloride disclose, with cod-liver oils giving a clear ultra-violet band at 328$\mu\mu$, a single sharp band at 604–608$\mu\mu$.

5. With crude cod-liver oils of high potency additional selective absorption between 565–585$\mu\mu$ is frequently observed in the blue solution.

6. The blue solutions given with concentrates have the main band at 620–624$\mu\mu$ and in many cases show a less intense band at 582–593$\mu\mu$.

7. Vitamin A is decomposed on treatment with sodium ethoxide. Concentrates so treated yield acids characterised by a series of well defined absorption bands with maxima near 394, 375, 350, 330, 316, 302, 282, 271 and 260$\mu\mu$.

8. Similarly absorbing acids are produced by the ordinary saponification process, but evidence is adduced showing that these acids cannot be ordinary fatty acids and that they are not present as simple glycerides in the oil itself.

We desire to express our thanks to Messrs Lever Brothers, Ltd., and Messrs Joseph Nathan and Co., Ltd., for material used in this investigation, and also to the Department of Scientific and Industrial Research for a grant to one of us (A. T.).

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V. SPECTROGRAPHIC DATA OF NATURAL FATS AND THEIR FATTY ACIDS IN RELATION TO VITAMIN A.

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The observation that vitamin A concentrates yield, on treatment with sodium ethoxide, acids of which the absorption spectra exhibit characteristic fine structure [Morton, Heilbron and Thompson, 1931] has prompted us to commence a detailed investigation of the absorption spectra of natural fats and their related acids.

In the present paper certain broad aspects of the problem are considered.

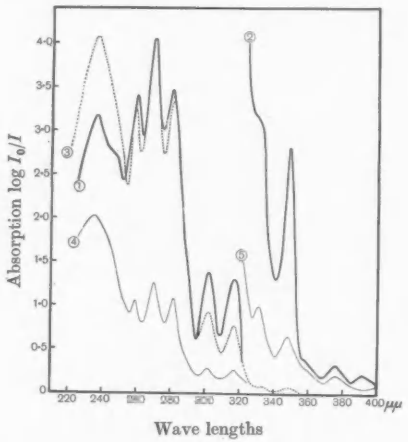

Fig. 1.

(1) Total acids from cod-liver oil 0·08 % [2 mm.].
(2) „ „ 1·00 % [2 mm.].
(3) Saturated and less unsaturated acids from cod-liver oil 0·4 % [2 mm.].
(4) Synthetic glycerides of cod-liver oil acids 0·08 % [2 mm.].
(5) „ „ „ 1·0 % [2 mm.].

Attention has not been solely confined to vitamin A-containing oils but has been extended to embrace typical vegetable fats. In the first place the total

acids[1] from cod-liver oil have been examined. The absorption spectra of these are characterised by sharp bands with heads at about 392, 375, 350, 330, 316, 302, 281, 270, 259 and 235$\mu\mu$ (Fig. 1, curves 1 and 2), and correspond closely with those of the selectively absorbing acids from the vitamin A concentrates [Morton *et al.* 1931, p. 24]. Fig. 2 (curve 2) gives the absorption spectrum of a

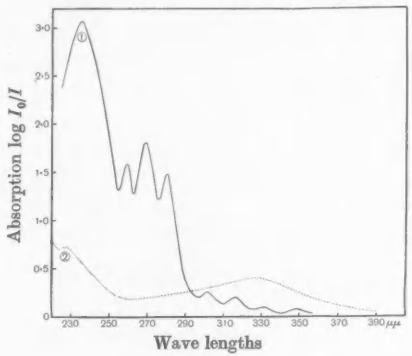

Fig. 2.

(1) —— Total acids from halibut-liver oil 0·2 % [2 mm.].
(2) ..... Halibut-liver oil 0·2 % [2 mm.].

halibut-liver oil rich in vitamin A (180 Carr and Price units) and here the smooth characteristic vitamin band at 328$\mu\mu$ is clearly shown with, in addition, a second small band at about 225$\mu\mu$. The acids obtained from this oil on saponification reveal on the other hand a wholly different type of absorption, bands with heads at identically the same positions as those of the cod-liver oil acids are now shown although a difference is observed in their relative intensities (Fig. 2, curve 1).

In Fig. 3 the absorption curve obtained for butter acids is given, butter being chosen to represent a non-liver animal fat containing vitamin A. It will be seen that five definite maxima appear, indicating the presence of small quantities of substances having the same bands as those shown by the cod- and halibut-liver oil acids. Turning now to butter-fat itself, this has been examined in chloroform solution and it is important to observe that in this case the spectrum shows distinct selective absorption at 322, 309, 284, 274 and 231$\mu\mu$. There is little doubt that these bands correspond with those of the acids in alcohol, the shift towards the red being a solvent effect quite similar to that observed by Morton and Heilbron [1930] in the carotene bands of the non-saponifiable matter from butter. It is important to note however that, in striking contrast to butter itself, its non-saponifiable fraction does not give rise to narrow ultra-violet absorption bands.

[1] Unless otherwise specified, total acids include unsaponifiable matter originally present in the oil, the acids being prepared in the usual way by alkaline hydrolysis of the fat followed by liberation of the acids and extraction of the latter with ether. In the case of the cod-liver acids, the removal of the unsaponifiable matter brought about no alteration in the spectrum of the acids.

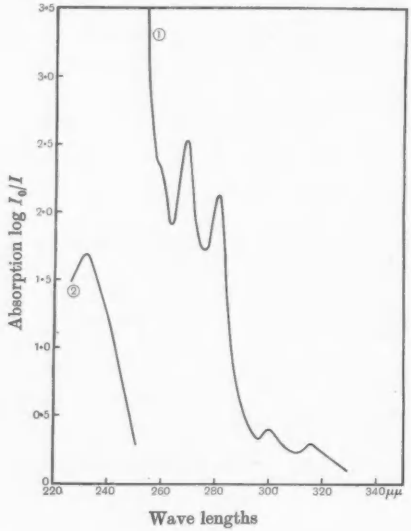

Fig. 3.

Total butter acids. (1) 1 % [1 cm.] alcohol. (2) 0·2 % [2 mm.].

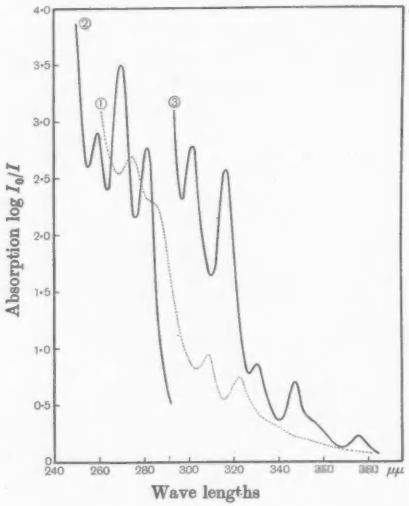

Fig. 4.

(1) Whale oil 0·234 mm.
(2) Acids from whale oil 0·25 % alcohol [5 mm.].
(3) „ „ 1·0 % alcohol [1 cm.].

The case of whale oil was next considered, this being selected as a typical marine animal non-liver oil deficient in vitamin A. The absorption spectra of the mixed acids from this oil are, as shown in Fig. 4 (curves 2 and 3), qualitatively, and roughly quantitatively, comparable with the cod- or halibut-liver acids. The oil itself (curve 1) like butter, also has selective absorption similar to that of the acids, but different in intensity. This specimen of whale-oil contained nearly 1 % of free fatty acids; in order to determine whether these were responsible for the observed selective absorption they were removed and the neutral oil was again submitted to spectrographic examination. The same banded structure was again observed, but the intensity of absorption in the region 260–280$\mu\mu$ appeared to be increased. It is thus clear that, as with butter, the selective absorption of the whale oil is not exclusively due to hydrolytic products of the fat.

In Fig. 5 (curves 1 and 2) the absorption spectra of the mixed fatty acids

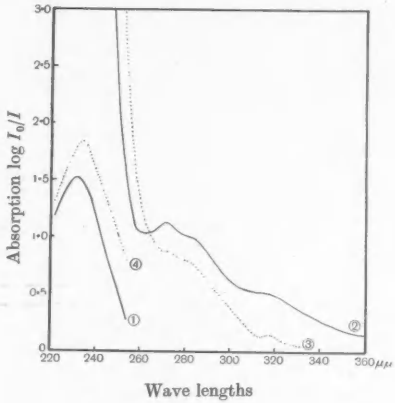

Fig. 5.

(1) Thresher shark-liver acids 0·2 % [4 mm.].
(2) ,, ,, 0·2 % [2 cm.].
(3) Methyl esters of Thresher shark-liver acids 0·2 % [4 mm.].
(4) ,, ,, ,, 0·04 % [4 mm.].

of a Thresher shark-liver oil, from which vitamin A is absent, are reproduced. A comparison of the curves with those given in Figs. 1, 2 and 4 affords evidence of marked differences in definition and intensity. In place of the numerous sharp bands in the latter curves there are here only ill-defined maxima at 270, 280 and 315$\mu\mu$; on the other hand, the prominent band at 230$\mu\mu$ still persists[1].

Finally we have selected two examples from the vegetable oil group. The absorption spectra of olive oil and its mixed acids are shown in Fig. 6, while

[1] It should be noted that a band of high intensity in the extreme ultra-violet occurs with great regularity in a wide range of unsaturated organic compounds.

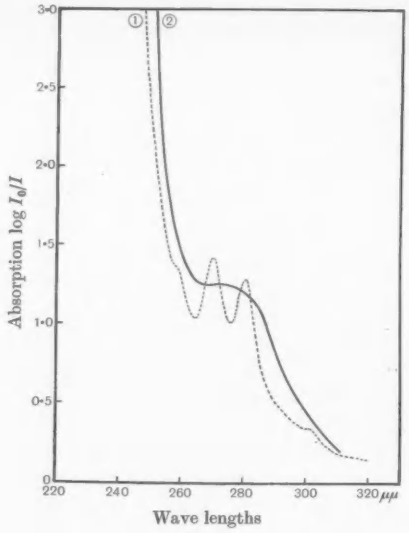

Fig. 6.

(1) ——— Olive oil acids [1 cm.] 1 % alcohol.
(2) - - - - - Olive oil [2 cm.] 1½ % alcohol.

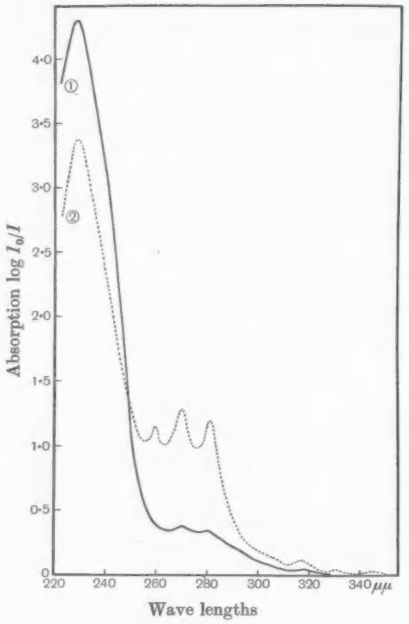

Fig. 7.

(1) ——— Cottonseed acids 0·2 % [3 mm.].
(2) - - - - - Cottonseed oil 1·0 % [2 mm.].

the curves for cottonseed oil and its acids are represented in Fig. 7. The curves differ markedly from those shown by the vitamin A-containing liver-oils and the corresponding acids of the latter; the vegetable oils exhibit a triplet group of bands with heads at 260, 271 and 281 $\mu\mu$ and indications of other absorption in the region 300–350 $\mu\mu$, but the typical vitamin A band is absent. The curves for the corresponding mixed acids somewhat surprisingly show less fine structure between 260–280 $\mu\mu$ than those for the respective oils, and thus stand in striking contrast with those of the fish-liver oil acids with which this discussion was opened.

Although the present communication is mainly preliminary in nature and confines itself for the most part to a statement of experimental observations, it is nevertheless desirable to consider the evidence available as to the nature of the compound or compounds which give rise to the characteristic elaborate spectrum with 8–10 sharply defined absorption bands. It seemed possible at one time that the source of the banded absorption might lie in some of the highly unsaturated fatty acids common to marine animal oils but the experiments now to be described lend little support to this view.

The total mixed fatty acids from cod-liver oil (Fig. 1) were separated into

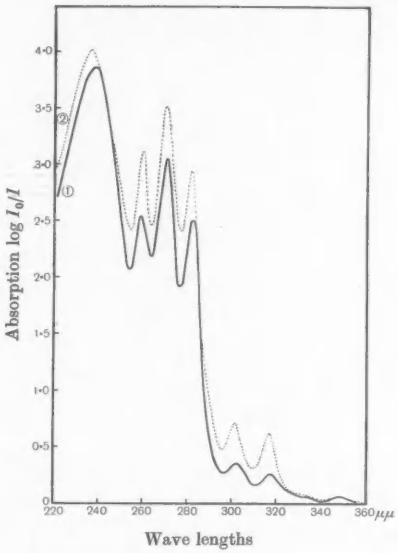

Fig. 8.

(1) ——— Highly unsaturated acids from cod-liver oil 0·06 % [2 mm.].
(2) - - - - - Methyl esters of above acids 0·05 % [2 mm.].

two groups by crystallisation of their lithium salts from acetone. The acids from the salts insoluble in acetone, which consist almost entirely of the

saturated and monoethylenic derivatives, have similar absorption (Fig. 1, curve 3) to that of the total mixed acids (Fig. 1, curves 1 and 2) but the intensity has been decreased by about 80 %. On the other hand with the acids from the soluble lithium salts which comprise the very highly unsaturated acids together with any unsaponifiable matter soluble in acetone, although the type of selective absorption is the same (Fig. 8, curve 1) the intensity is greater than with the total mixed acids. Fig. 8 (curve 2) shows the absorption curve of the corresponding methyl esters from the soluble lithium salts; this is in all respects similar to the curve of the free acids. These esters were submitted to fractionation under high vacuum as follows:

Fraction	Wt (g.)	B.P. (1 mm.)	Sap. equiv.	Iodine value
1	15·5	80–160°	283·8	138·2
2	41·6	160–177°	294·8	208·1
3	31·9	177–185°	307·0	266·4
4	46·2	185–190°	319·9	271·3
5	59·3	190–195°	333·7	285·4
6	25·4	195–200°	345·7	271·0
*Residue	84·0			

* Mainly polymerised highly unsaturated esters.

and the absorption spectrum of each of these primary fractions was observed. The following table gives the approximate relative intensity of the various bands calculated to 1 % solution in alcohol and 1 cm. thickness.

Wave length of maxima ...	235 $\mu\mu$	270 $\mu\mu$	281 $\mu\mu$	316 $\mu\mu$
Fraction 1	100	42	25	8
„ 2	150	25	16	2
„ 3	200	35	28	?
„ 4	212	75	53	2
„ 5	250	35	28	3
„ 6	325	36	25	4

It will be observed that whereas the intensity of the band at 235 $\mu\mu$ (*cf.* footnote, p. 33) rises steadily throughout the series to a maximum, the bands at 270 and 281 $\mu\mu$ reach maximum intensity in fraction 4, but this intensity is already relatively much lessened as compared with that of the undistilled esters. Fraction 4 was re-distilled and the two main fractions having B.P. respectively 160–170° and 170–172°, and corresponding iodine values 279·8 and 301·5, were again examined. The absorption curves of these fractions are reproduced in Fig. 9 (curves 2 and 3). Comparison with the absorption of the esters prior to fractionation (curve 1) shows that a pronounced decrease in both absorption and selectivity has been brought about, presumably by decomposition. In view of these results it is improbable that the recorded fine structure can be attributed to esters of highly unsaturated acids ("clupanodonic" type). Moreover, a similar even more unsaturated re-fractionated ester from the acids of Thresher shark-liver oil (sap. equiv. 327·3, iodine value 320·3) fails to show (Fig. 5, curves 3 and 4) the characteristic fine structure under discussion.

It might be argued that these results leave open the bare possibility that the observed selective absorption may be due to the presence of those highly

unsaturated esters of cod-liver oil which polymerise entirely [*cf.* Guha, Hilditch and Lovern, 1930], and which are left in the residue (84 g.) from the primary fractionation. If these were indeed the cause it is difficult to understand why the original oil is wholly devoid of the banded spectrum for if the acids were actually present in the oils themselves in the form of glyceryl esters there is no reason to suppose that, with glyceryl as with methyl esters, the same absorption should not be shown. We have in fact observed that the neutral triglycerides prepared from glycerol and the total mixed fatty acids of cod-liver oil by heating the alcohol with an excess of the free fatty acids under reduced pressure exhibit qualitatively the same absorption characteristics as the original fatty acids (Fig. 1, curves 4 and 5).

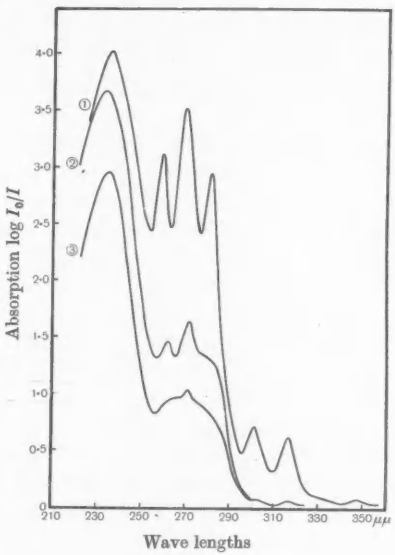

Fig. 9.

(1) Methyl esters of highly unsaturated cod-liver oil acids 0·05 % [2 mm.].
(2) After refractionation; iodine value = 279·8 } 0·05 % [2 mm.].
(3) " " " = 301·5 }

Discussion of results and summary.

In considering the data, a true perspective must be maintained. From the physiological angle vitamin A, vitamin D and ergosterol loom large, but from the point of view of quantitative analysis they are merely contaminants of minor constituents. This background cannot be dispensed with when the investigation of liver oils broadens out to embrace the indirect as well as the direct attack on the nature of the substances to which we attach special biological significance. Whilst the results now recorded form a logical sequence of the study of vitamin A, and it is natural to hope that a not very remote

connection may emerge, there seems to be no justification for allowing the discussion to be dominated by vitamin A.

It appears to us at the moment that the simplest explanation in consonance with all the facts so far observed is that under hydrolytic conditions a substance (or substances) accompanying vitamin A gives rise to acid decomposition products which display intense selective absorption. We suggest that even under the ordinary mild conditions of saponification, sufficient of this acidic decomposition product is formed to give rise to the observed spectra. That the acid decomposition products are in some way connected with the presence of vitamin A is indicated by the distinctly different absorption curves obtained with the acids from the non-vitamin A-containing olive or cottonseed oils.

It must again be emphasised that the absorbing acidic substances could not have been present originally in the form of simple esters for, were this the case, the liver oils should exhibit similar selective absorption. Indeed, once the acidic substances have been developed their characteristic spectra persist after their esterification with either methyl alcohol or glycerol. We are thus forced to the conclusion that their formation is the result of some form of molecular breakdown.

A most interesting corollary lies in the observations connected with the whale (blubber) oil examined and also with butter. With whale oil we are dealing with a vitamin-free fat which nevertheless originates in the liver, from which organ it is highly improbable that the vitamin is absent. If it be assumed that products of both hydrolytic and synthetic actions taking place in the liver are deposited in the tissue fat, an explanation is immediately forthcoming of the selective absorption of the oil as well as its derived acids. The same reasoning appertains in the case of butter, although here vitamin A itself is also present to a small extent in the milk fat.

The correctness of the foregoing speculations remains to be tested by the extended experimental work which we are undertaking on this subject. The main object of the present communication has been to record the evidence which we have collected showing that the fatty acids (or esters prepared therefrom) produced in the ordinary hydrolysis of vitamin A-containing liver oils, or of some other fatty oils from animals whose liver oils contain vitamin A, yield highly characteristic banded absorption spectra which are absent from the corresponding acids of vitamin A-free oils.

We desire to thank Dr G. Collin, Mr K. D. Guha and Dr A. Thompson for assistance in the preparation of a number of the compounds studied in the course of this work and the Department of Scientific and Industrial Research for assistance in various directions.

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VI. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XIX. 6-HYDROXY-2-METHYLBENZOIC ACID, A PRODUCT OF THE METABOLISM OF GLUCOSE BY *PENICILLIUM GRISEO-FULVUM* DIERCKX.

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(*Received November 28th, 1930.*)

In a series of publications which have recently appeared [Raistrick *et al.*, 1931] an account is given of an intensive study, extending over a period of seven years, of the products of the metabolism of glucose, in synthetic media containing no other source of carbon, by a large number of different species of the lower fungi. Several new types of metabolic products were described, many of which were shown to contain a benzene nucleus.

More recently it has been possible to extend this work, and, thanks to the courtesy of Professor Philip Biourge of the University of Louvain, several different species of *Penicillium* have been examined which were not available previously.

One of these species was *P. griseo-fulvum* Dierckx, which was originally described by Dierckx [1901], and, more recently, by Biourge [1923] in his monograph on the genus *Penicillium*. This species bears Biourge's catalogue number 34. In view of the interesting results we obtained with this species, we asked Professor Biourge to re-examine our culture and his report follows.

"Voici assez parfaitement déterminé le *Penicillium* que vous m'avez envoyé pendant les vacances. C'est bien le *Penicillium griseo-fulvum* Dierckx (Biourge No. 34). C'est une espèce peu commune. C'est la troisième fois que je la rencontre, y compris l'observation originale de Dierckx en 1898–1900, dans mon laboratoire. Elle est bien pure. Spores bleu céleste très pâle, et finalement gris-rosé. Revers jaune à jaune-rouge. Coremia tardifs, liquéfaction de la gélatine souvent précoce, odeur 0."

We desire to thank Professor Biourge for his friendly co-operation.

It was found that, when this species of *Penicillium* is grown on a synthetic medium containing glucose as the sole source of carbon, the metabolism solution, after some days, gives an intense purple colour with ferric chloride, very similar in shade to aqueous potassium permanganate solution. In addition the metabolism solution also gives a heavy, pale yellow precipitate with

bromine water. The isolation and identification of the substance responsible for these reactions is the object of the present communication.

Preparation of the metabolic product.

A quantity of a slightly modified Czapek-Dox medium was made up, having the following composition:

$NaNO_3$	2·0 g.
KH_2PO_4	1·0 g.
KCl	0·5 g.
$MgSO_4.7H_2O$	0·5 g.
$FeSO_4.7H_2O$	0·02 g.
Glucose (pure)	50·0 g.
Dist. water	to 1000 cc.

350 cc. quantities of this medium were placed in 67 1-litre conical flasks, which were plugged with cotton-wool, and sterilised by steaming for half an hour on each of three consecutive days. Each flask was heavily sown with an emulsion, in the above medium, of spores prepared from a 17-day culture of *P. griseo-fulvum* Dierckx, grown on Czapek-Dox agar at 25°. The flasks were thoroughly shaken and incubated at 25° for 35 days. At the end of this period the surface of the medium was completely covered with a thick mycelial felt, showing areas of pale green sporing patches, the intervening spaces consisting of sterile mycelium. The reverse of the mycelium was brick-red in colour. The contents of all the flasks were now filtered without previous sterilisation, the mycelium pressed and dried *in vacuo* at 50°. The combined filtrates, which were orange-yellow in colour, had the following characteristics.

(1) Glucose (by polarimeter) 0·254 %.

(2) Titratable acidity = 2·13 cc. *N* acid per 250 cc. medium.

(3) Bromine absorption (by Koppeschaar's method) = 3·8 mg. per cc.

(4) The metabolism solution gave a very intense purple colour with aqueous ferric chloride and a heavy pale yellow precipitate with bromine water.

The whole of the filtrate was acidified with 400 cc. of 2*N* H_2SO_4 and shaken with half its volume of ether, by which means the substance responsible for the ferric chloride reaction was completely extracted. The ethereal solution was washed with a little water, filtered and evaporated to dryness. The dry residue, which was brownish in colour and completely crystalline, weighed 26·9 g. corresponding to a yield of 2·42 % of the glucose consumed. It was crystallised from chloroform, from which solvent it separated in long white needles, which were practically pure but were recrystallised several times for analysis.

The dried mycelium was powdered and exhaustively extracted with ether. The ethereal extract on evaporation deposited a quantity of a white crystalline material which melted in a crude state at about 150°, gave a brownish purple

colour with ferric chloride, but is apparently not identical with the product extracted from the metabolism solution. The nature of this product will form the subject of a future communication.

Properties, analysis and derivatives of the metabolic product.

The product extracted from the metabolism solution and crystallised from chloroform has the following properties. It consists of beautiful white needles melting without decomposition at 170–171° and may be readily sublimed unchanged in a high vacuum. It is not very soluble in cold water, but readily dissolves in hot water from which it separates on cooling in long white needles. It is very soluble in ether and alcohol, moderately soluble in hot, but not very soluble in cold, chloroform. Its solution in water or alcohol gives a very intense purple-violet colour with ferric chloride, very similar to that given by salicylic acid. Its aqueous solution gives an immediate precipitate with bromine water, and reacts acid to litmus. It gave the following results on analysis:

C, 62·91 and 63·02 %; H, 5·28 and 5·33 % (theoretical for $C_8H_8O_3$, C, 63·13; H, 5·30 %).

In an estimation of the molecular weight by the Rast camphor method, 0·217 mg. lowered the M.P. of 2·608 mg. camphor 20·2°, corresponding to a molecular weight of 160 (theoretical for $C_8H_8O_3$, 152).

Titration with $N/10$ sodium hydroxide to phenolphthalein gave an equivalent of 152·6 (theoretical for $C_8H_8O_3$, assuming this to be a monobasic acid, 152).

Methyl ether. 3 g. of the metabolic product were methylated by shaking with 9 cc. of dimethyl sulphate and an excess of 10 % sodium hydroxide. The mixture was maintained at room temperature for about 2 hours and was then boiled under a reflux condenser until perfectly clear. The solution was cooled, filtered, acidified and extracted with ether. The ethereal solution was evaporated to dryness and the dry residue, which weighed 3·1 g., was crystallised several times from boiling water. The crystallised material consisted of colourless, elongated hexagonal prisms (plates), which melted at 139° and gave no colour with ferric chloride. It gave the following results on analysis:

C, 65·39 %; H, 5·97 % (theoretical for $C_9H_{10}O_3$, C, 65·03 %; H, 6·07 %).

A Zeisel estimation gave 18·66 % OCH_3 (theoretical, 18·68 %).

Titration with $N/10$ sodium hydroxide to phenolphthalein gave an equivalent of 166·3 (theoretical for $C_9H_{10}O_3$, assuming this to be a monobasic acid, 166·1).

Acetyl derivative. 2 g. of the metabolic product, 3·3 cc. acetic anhydride and 6·6 cc. pyridine were mixed and incubated in a closed flask at 37° for 4 days. The mixture was cooled, diluted with 100 cc. of water, acidified to Congo red with $2N$ H_2SO_4 and extracted with ether. The crystalline residue from the ether, weighing 2·5 g., was recrystallised several times from boiling

benzene, from which solvent it separated in colourless, elongated prisms, which melted without decomposition at 131°. This product which gave no colour with ferric chloride gave the following results on analysis:

C, 62·29 %, H, 5·23 % (theoretical for $C_{10}H_{10}O_4$, C, 61·83 %; H, 5·19 %).

0·2044 g. titrated with $N/10$ sodium hydroxide to phenolphthalein required 10·40 cc. giving an equivalent of 196·5 (theoretical for $C_{10}H_{10}O_4$, assuming this to be a monobasic acid, 194·1).

On account of the volatility of the metabolic product in steam, it was impossible to carry out a direct estimation of the acetyl groups, but this was done indirectly as follows. An excess of $N/10$ sodium hydroxide was added to the above neutralised solution, the mixture boiled for 3 hours under reflux, cooled and the excess of alkali titrated with $N/10$ hydrochloric acid. Acidity equivalent to 10·98 cc. $N/10$ was produced during the hydrolysis, from which it is evident that the derivative is a monoacetyl compound.

Constitution of the metabolic product.

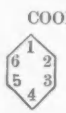
The above results indicate that the metabolic product has the empirical formula $C_8H_8O_3$, contains one hydroxyl group, which is probably phenolic in nature because of the purple colour produced with ferric chloride, and a carboxyl group. Since the material in aqueous solution also gives an insoluble bromine compound with bromine water, it is probably a benzene derivative.

Accepting this assumption for the moment, the metabolic product must be either (i) a hydroxyphenylacetic acid *or* (ii) a hydroxymethylbenzoic acid.

The possibility of the metabolic product being a phenylglycollic acid and having the hydroxyl group in the side chain is ruled out because of the reaction with ferric chloride.

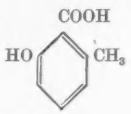
(i) The three possible hydroxyphenylacetic acids, 2-hydroxy-, 3-hydroxy- and 4-hydroxy- are known and melt respectively at 145–147°, 129° and 148°. The metabolic product melts at 171° and this, together with its general properties, proves that it is not a hydroxyphenylacetic acid.

(ii) There are ten possible hydroxymethylbenzoic acids, which have all been described. Writing the formulae for these according to the scheme



the metabolic product cannot be identical with any of the following, since they give no colour with ferric chloride; 5-OH 2-CH_3; 4-OH 2-CH_3; 3-OH 2-CH_3; 5-OH 3-CH_3; 4-OH 3-CH_3; 3-OH 4-CH_3. This leaves the possibility that the metabolic product is one of the four methylsalicylic acids; 6-OH 2-CH_3, 2-OH 3-CH_3, 6-OH 3-CH_3, 2-OH 4-CH_3. All these acids give an intense violet colour with ferric chloride and melt respectively at 168–169°,

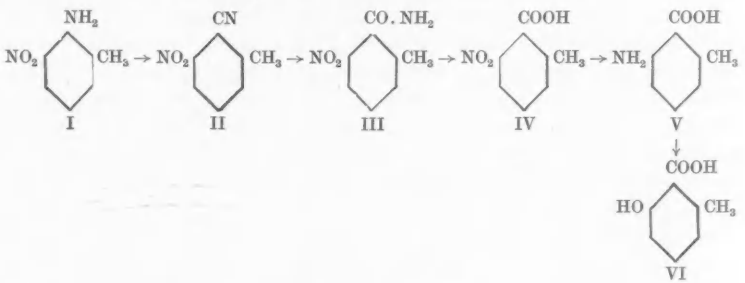
167°, 153° and 177°. (The highest recorded melting point is given in each case.) These melting points are not sufficiently far removed from the melting point of the metabolic product (171°) to be definitive. The melting points of the methyl ethers are, however, very much more widely separated and are respectively 139°, 85°, 70° and 104°. Since the melting point of the methyl ether of the metabolic product is 139°, it appears probable that the metabolic product is identical with 6-hydroxy-2-methylbenzoic acid of the following formula.



This conclusion was confirmed by synthesis.

Synthesis of the metabolic product.

We are indebted to Dr Alexander Robertson for synthetic specimens of 6-hydroxy-2-methylbenzoic acid, of its methyl ether and of its acetyl derivative. These were prepared as follows:



The nitrotoluic acid (IV) was prepared from the nitrotoluidine (I), *via* the nitronitrile (II), and nitroamide (III), according to the methods of Gabriel and Thieme [1919] and Kenner and Witham [1921].

The nitro-acid (IV) was reduced by means of ferrous sulphate and ammonia as used by Gabriel and Thieme. The amino-acid (V) was isolated by extraction with ether, and was converted into the hydroxy-acid (VI) without further purification. A solution of the amino-acid (6 g.) in 90 cc. *N*/2 HCl was cooled to 0°, treated with sodium nitrite (3 g.) and the mixture kept at 0° for 1 hour. 5 % sulphuric acid (40 cc.) was then added and the mixture was rapidly heated to 65° by plunging the vessel into a water-bath at 70–75°, and was maintained at this temperature for $\frac{1}{4}$ hour. The hydroxy-acid, contaminated with only traces of brown coloured material, quickly separated. Concentrated hydrochloric acid (40 cc.) was added to the cooled reaction mixture, and after 2 hours in the ice-chest the hydroxy-acid was collected, washed with water, and air-dried. Yield 3–3·5 g. It was purified by crystallisation from chloroform.

The synthetic hydroxy-acid gives the same colour reaction with ferric chloride as the metabolic product, has the same crystalline form, and the same melting point (170–171°). A mixture of the natural and synthetic products melts at the same temperature.

The methyl ether and the acetyl derivative of the synthetic hydroxy-acid were prepared by the methods previously described for the corresponding derivatives of the metabolic product.

The melting point of the methyl ether of the metabolic product, of the synthetic acid and of a mixture of the two was found to be 139° in all three cases. Similarly, the melting point of the acetyl derivative of the metabolic product, of the synthetic acid and of a mixture of the two was found to be 131° in all three cases, and hence identity of the metabolic product with the synthetic acid (6-hydroxy-2-methylbenzoic acid) is clearly established.

Identity of bromine compound.

It has been previously mentioned (p. 40) that the metabolism solution gives a heavy pale yellow precipitate with bromine water. In order to prepare a quantity of this material 5 litres of metabolism solution were treated with a slight excess of a saturated solution of bromine in water, allowed to stand overnight, and the precipitate filtered off. It was air-dried and extracted with ether. The ether solution on evaporation left a brownish residue consisting of rosettes of needles. A portion of this was recrystallised from light petroleum and then sublimed in a mercury vapour vacuum, giving a sublimate, at a bath temperature of 50–60°, of pale yellow needles, which melted at 84° and on admixture with a sublimed synthetic specimen of 2:4:6-tribromo-*m*-cresol had a mixed melting point of 83–84°.

It contained 69·6 % Br (theoretical for $C_7H_5OBr_3$, 69·5 %). Since the same substance is produced when bromine water is added to a dilute aqueous solution of the sodium salt of the metabolic product $C_8H_8O_3$, it is evident that this material is responsible for both the purple colour with ferric chloride and for the precipitate with bromine water given by the metabolism solution. This precipitate consists principally of 2:4:6-tribromo-*m*-cresol.

SUMMARY.

6-Hydroxy-2-methylbenzoic acid was isolated and identified as a product of the metabolism of glucose by *Penicillium griseo-fulvum* Dierckx.

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VII. ON THE BEHAVIOUR OF "INDIFFERENT" ELECTRODES WHEN USED FOR THE DETERMINATION OF OXIDATION-REDUCTION POTENTIALS IN THE PRESENCE OF HYDROGEN.

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(*Received December 3rd, 1930.*)

In some observations on the reduction potential of cultures of anaerobic organisms recently reported [Lepper and Martin, 1930] we found that gold electrodes gave entirely different results from iridium electrodes if hydrogen was liberated during growth. The gold electrode remained poised by the feeble parahaematin-haemochromogen oxidation-reduction system present in the muscle fibres of the meat used in the medium. The iridium electrodes, on the other hand, were rapidly converted into hydrogen electrodes and reached the potential of the buffer solution surrounding the meat. In order to get a better understanding of the behaviour of these electrodes we have examined their potential in weakly poised and unpoised buffer solutions in the presence of nitrogen, hydrogen and oxygen.

Preparation of the electrodes.

The iridium electrodes were prepared by the method of Westhaver [1905], the glass being given two coats of iridium.

The gold electrodes were made by heavily gilding platinum plates electrolytically in a bath of gold cyanide. As soon as the platinum appeared to be well gilded the electrode was removed from the bath, dried and heated in the blowpipe. This process was repeated four or five times until the electrode was a deep gold colour after heating. By this means a substantial film of gold was alloyed on to the platinum before it was finally coated with electrolytically deposited gold. Electrodes made in this way were much less sensitive to hydrogen and oxygen than merely gilded platinum electrodes.

Experiments in weakly poised solutions.

The electrodes were placed in phosphate mixtures containing indigo-carmine of different molecular strengths. The air was expelled from the electrode vessel by nitrogen deprived of traces of oxygen by passing through a

solution of anthraquinol made by reducing an alkaline solution of anthraquinone with sodium hydrosulphite [Conant and Fieser, 1924]. To secure fine bubbles the gas was made to traverse a piece of kieselguhr filter candle as shown in Fig. 1. When the air had been displaced from the phosphate solution a few drops of weak sodium hydrosulphite were added from a burette to reduce the indigo-carmine in part and readings of the potential were taken until it had become steady. Hydrogen free from oxygen was now run through the apparatus until the change in potential of the electrodes which took place had again reached its limit.

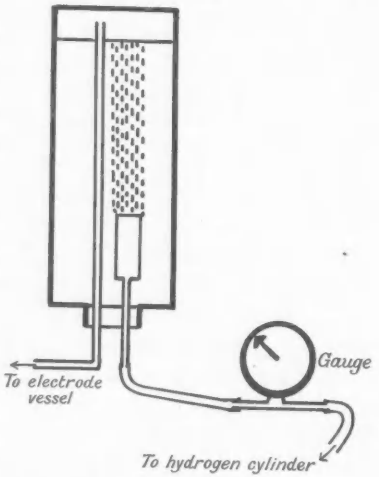

Fig. 1. Wash bottle for removal of oxygen from nitrogen and hydrogen.

When the concentration of indigo-carmine was 0·001 *M* the gold electrode remained perfectly stable on substituting hydrogen for nitrogen, but in a concentration of 0·0001 *M* the potential rose 8 mv.

With a concentration of 0·002 *M* indigo-carmine the iridium electrode showed a rise of 10 mv. and with a concentration of 0·0001 *M* the electrode had nearly reached the potential of the hydrogen electrode by the end of half an hour. The behaviour of the iridium electrode is shown in Fig. 2.

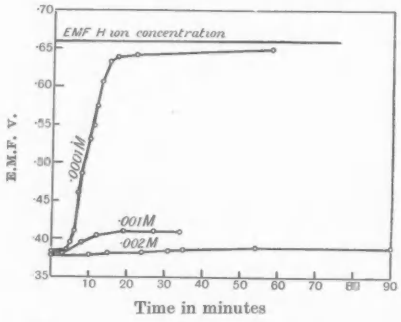

Fig. 2. The effect of hydrogen on an iridium electrode in different concentrations of indigo-carmine.

Experiments with unpoised buffer solutions.

A phosphate mixture was placed in the electrode vessel and the air was expelled by nitrogen. When sufficient time had been given to remove all the

oxygen from the solution hydrogen was substituted. At the end of $1\frac{1}{4}$ hours the negative drift of the gold electrode was 45 mv., whereas the iridium electrode had reached the potential corresponding to the hydrogen ion concentration of the solution, $-0{\cdot}7155$ v., where it remained steady (Fig. 3).

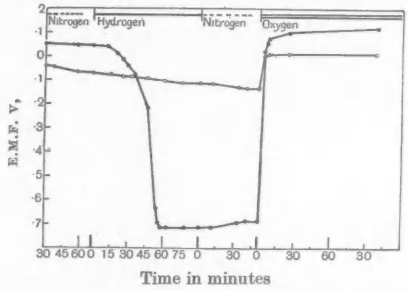

Fig. 3. The effect of hydrogen and oxygen on gold and iridium electrodes in phosphate buffer p_H 8·0.

•——•——• Iridium. o——o——o Gold.

The hydrogen was then displaced by nitrogen. After 45 minutes the gold showed a potential increase of 16 mv. and the iridium had fallen but 28 mv., indicating that the hydrogen absorbed by the iridium electrode is given off to an atmosphere of nitrogen only very slowly.

Lastly, when oxygen was run through the solution a rapid fall of potential occurred with both electrodes. At the end of 30 minutes the gold electrode again gave a steady potential. The iridium, on the other hand, after the first sudden fall to $+0{\cdot}1$ v. continued to show a slow positive drift throughout the 5-hour period of observation.

The maximum potential of $-0{\cdot}125$ v. observed at the gold electrode during the second period with nitrogen probably represents closely the potential of a really indifferent electrode at this p_H, as the oxygen present in the electrode at the time of immersion would, we presume, have been removed by the hydrogen.

It can be seen from Fig. 3 that gold appears to be much more sensitive to oxygen than it is to hydrogen.

Discussion.

The explanation of the different behaviour of electrodes of gold and iridium during the growth of microbes when hydrogen is evolved appears to be that, the evolution of hydrogen being occasioned by an irreversible process and gold being relatively insensible to hydrogen, there is no reversible oxidation-reduction system present with a more negative range than the parahaematin-haemochromogen with which the gold electrode can come into electro-chemical equilibrium.

This interpretation is supported by the fact that when the more electronegative dye safranine was added to the culture medium so as to make a concentration of about 0·01 %, in 1 hour after inoculation with *B. welchii* the E.M.F. afforded by the iridium electrode fell as before to that of the hydrogen electrode, but the potential of the gold electrode, instead of remaining poised by the parahaematin-haemochromogen dropped steeply until it was poised by the safranine around −0·33 v.

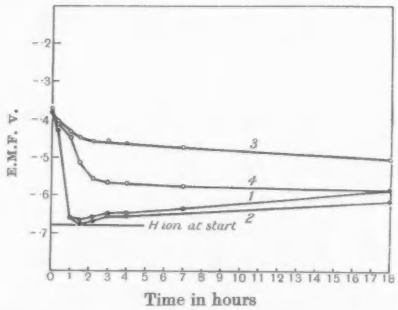

Fig. 4. The behaviour of gold and iridium electrodes when *B. welchii* grows in meat medium.
Curve 1. Iridium in meat. Curve 2. Iridium in meat containing safranine.
Curve 3. Gold in meat. Curve 4. Gold in meat containing safranine.

The iridium electrode behaves otherwise, because it becomes charged with hydrogen from the solution and functions as a hydrogen electrode. The reason for the hydrogen overvoltage noticed by Gillespie [1920] and also by Cannan, Cohen and Clark [1926] which also occurred in some of our experiments, is not obvious. The gas evolved contains less than 50 % hydrogen, but the conditions under which it is formed are conducive to supersaturation of the solution. Moreover, it is atomic hydrogen which is first separated, and this may find direct access to the electrode.

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VIII. THE FATTY ACIDS OF THE CAT'S KIDNEY. I.

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From the Department of Physiology, University of Sheffield.

(*Received December 3rd, 1930.*)

Introduction.

When the fatty acids which can be isolated from the cat's kidney are compared with those from the kidneys of other animals it is found that they present some striking differences. Whereas the mixed fatty acids from sheep or ox kidneys amount to about 2 % of the weight of the fresh organ and possess an iodine value of approximately 135, cat's kidneys yield about 4 % of fatty acids with an iodine value in the neighbourhood of 60. In spite of their lower iodine value, however, these mixed acids are semi-liquid whilst the more unsaturated acids from other animals are quite solid at room temperatures.

The earliest recorded values are those of Leathes and Meyer-Wedell [1909]. They found that the iodine values of fatty acids from the kidneys of normal cats were from 60 to 70, almost identical, that is, with the iodine value of 65 for the fatty acids from the adipose tissue fat. Mottram [1916] found that the average amount of fat in the kidneys of eight well-fed healthy cats was 5·11 % with an average iodine value for the fatty acids of 56, actually lower than the average iodine value of 61·4 for the adipose tissue fatty acids from the same cats. He also observed that the kidney tubules were heavily lined with stainable fat. Mottram suggested that the abnormally low iodine values were the result of an infiltration of fat into the kidneys, but for reasons which will be discussed later it seems improbable that this can be the correct explanation.

The kidney fat of the cat has been re-investigated with the results which are now to be described.

Experimental.

Fatty acid estimations.

The low iodine values reported by Leathes and Meyer-Wedell [1909] and Mottram [1916] have been confirmed. It should be noted that Mottram's values refer only to the cortex of the kidney, but the following results show that the same is true for the whole kidney. The percentage of fatty acids in the kidneys was determined by the method described by Leathes and Raper 1925] and the iodine values were determined by the method of Wijs. Each

value given was obtained from the whole of one kidney; where two values are given they refer to left and right kidneys respectively. The iodine values were determined immediately after isolation of the fatty acids.

Table I.

Fatty acids (%)	Iodine value	Fatty acids (%)	Iodine value
3·72 3·69	82·6	6·37 6·83	71·7
4·28 4·61	84·1	5·70 5·48	71·0
5·40 6·54	69·3	3·65 3·78	87·7
4·14 4·37	80·5	3·21	97·8
6·77 6·73	69·6	5·68	76·0
5·58	63·5	2·75	113·0
5·63 5·92	69·5	2·16	98·0
6·30 6·18	88·7	4·15	79·0
5·29 4·76	79·9	3·58	87·4
4·07 4·06	86·5	6·83	53·2

Fatty acids: Max. value = 6·83 %.
Min. value = 2·16 %.
Mean of 32 = 4·9 %.

These values may be compared with those from typical beef kidney which had 2·09 % and 2·02 % fatty acids with corresponding iodine values of 133·8 and 131·0; and with the average for 34 human kidneys examined by Imrie [1914] of 1·9 % with iodine value 131.

Examination of the Fatty Acids.

A. *The isolation of the mixed fatty acids.*

The kidneys were collected from a large number of cats during a period of several months. Immediately after death the kidneys were removed from the body, carefully freed from all visible fat and connective tissue, weighed, cut into slices and placed in a small porcelain basin. The tissue was then saponified with a 50 % aqueous solution of caustic potash until completely liquid, methylated spirit was then added and heating continued until hydrolysis was complete. The resulting soap solution was poured into a stock-bottle. When about a kilogram of kidneys had been collected and saponified in this way, the accumulated soap solution was made strongly acid with 40 % sulphuric acid. After dilution with water to a suitable volume it was extracted three times with light petroleum. The extracts, which were deep red in colour, were collected in a flask and the solvent was removed on the water-bath in a slow current of carbon dioxide, the last traces being removed *in vacuo*. The crude fatty acids remaining in the flask were freed from unsaponifiable matter as completely as possible by repeatedly shaking out their solution in potassium hydroxide with light petroleum. The aqueous fluids were again acidified with sulphuric acid and extracted with light petroleum, the fatty acids being recovered by evaporation of the dried extract in a stream of carbon dioxide to prevent oxidation.

The mixed fatty acids formed a semi-liquid mass, yellow-brown in colour. If the flask containing them was left for some days in a tilted position, a liquid portion drained away from the solid acids. The whole readily liquefied

on very gentle warming. A peculiar musty smell was invariably associated with the mixed acids, though this may have been due to the presence of small amounts of impurities. The mixed acids usually had an iodine value about 56 and M.W. by titration about 274.

B. *Separation of the mixed fatty acids.*

The mixed fatty acids were separated into a liquid and a solid fraction by means of the lead soap-ether process.

The results of three distinct separations are summarised in Table II.

Table II. *Results of the separation of the fatty acids by the lead soap-ether process.*

	Mixed acids		Unsaturated acids			Saturated acids	
Batch	Iodine value	M.W.	Iodine value	M.W.	% of mixed	Iodine value	M.W.
A	56·9	264	62·5	263	61·5	4·4	—
B	56·4	274	67·3	274·2	60·6	5·8	276·4
C	56·4	274	68·7	282·4	63·9	14·0	275

The solid fraction (*lead soaps insoluble in ether*). These fatty acids were quite hard and solid, looking exactly like stearic acid. The M.W. of 274 combined with the very low iodine value suggests that they are mainly a mixture of palmitic and stearic acids in about equivalent proportions.

This is confirmed by the fact that an acid was obtained by repeated crystallisation of these acids from alcohol which had iodine value 0, M.W. 283·6 and M.P. 68·5°. 0·2280 g. acid neutralised 8·04 cc. *N*/10 NaOH; equivalent wt., 283·6. Stearic acid requires equivalent wt. 284, M.P. 69·2°, iodine value 0.

The liquid fraction (*lead soaps soluble in ether*). The acids composing this fraction were quite liquid even after standing for many months, although the iodine value was only 67·5. They were deep red in colour, but by boiling with charcoal in light petroleum solution they were finally obtained as a pale yellow oil. The peculiar musty odour was still apparent.

C. *Further investigation of the liquid fraction.*

The fluidity of this fraction combined with the very low iodine value (67·5) suggested the possible presence in the mixture of a liquid saturated acid. If this view were correct it should be theoretically possible to separate it from the liquid unsaturated acids by a preliminary hydrogenation of the mixture, by which these latter acids would be turned into solid saturated acids, followed by the usual lead soap-ether separation.

Hydrogenation of the liquid fraction. The hydrogenation was effected by the use of a platinum catalyst prepared by the method of Voorhees and Adams [1922]. This was found to be superior to a palladium hydrosol prepared by the method of Paal [1902, 1904]. Before the catalyst can be used for hydrogenations it must first be converted from the finely divided platinum

oxide into finely divided platinum black. A small quantity of the platinum oxide catalyst is shaken into a suspension in alcohol in a small round flask, the air is then swept out by a current of hydrogen from a Kipp's apparatus, and the suspension shaken in an atmosphere of hydrogen. In a short time a perfectly black, fine suspension is obtained. The fatty acids dissolved in a small volume of alcohol are then added to the flask containing the platinum-black catalyst and shaken for 2–3 hours in a hydrogen atmosphere under slightly more than atmospheric pressure. 26 g. of mixed liquid acids (iodine value 67·5) were in this way converted into 25 g. of hydrogenated acids which had the iodine value 7·8 and were quite solid.

Separation of the hydrogenated acids. The 25 g. of hydrogenated acids were next separated by the lead soap-ether process into (1) *a solid fraction*—a white hard solid, M.P. 69°, iodine value 0, M.W. 283·9, which therefore consists of pure stearic acid formed by hydrogenation of oleic and linoleic acids present in the liquid acids, and (2) *a liquid fraction*—7·5 g. of pale yellow mobile oil, having the iodine value 11·3, M.W. 268.

Esterification of the liquid acids. The 7·0 g. of liquid acids were dissolved in 50 cc. of methyl alcohol containing 3 cc. of sulphuric acid and heated on a water-bath for 2 hours. The liquid was then poured into a large excess of water and the esters were shaken out with ether. The ethereal extract was washed with water, then with potassium carbonate solution, again with water and finally dried over calcium chloride. Removal of the ether left 6·8 g. of a pale yellow, almost odourless, mobile oil.

The methyl esters were then distilled under a pressure of 15–20 mm. The first fraction distilled mainly at 105–115°, the temperature then rose fairly rapidly to 135° and the remainder of the esters distilled at 135–140°. The distillates were colourless faintly fatty-smelling oils. A dark brown, very viscous oil remained as a residue in the distilling flask, which after boiling for 2 hours with 20 % alcoholic potash remained unchanged. It was soluble in hot alcohol but insoluble in light petroleum or water.

Saponification of the ester fractions. The ester fractions were hydrolysed separately by boiling for 1–2 hours with 10 % alcoholic caustic potash. The resulting soap solutions were then acidified with H_2SO_4 and shaken out with light petroleum, and the extract was washed with water and dried. The fatty acids obtained on removing the solvent were from each fraction colourless, odourless, mobile oils.

Fraction (1). Wt. 1·2 g.

0·1938 g. neutralised 8·55 cc. *N*/10 NaOH. Equivalent wt. = 226·6.

0·1900 g. absorbed 0·2 mg. I_2. Iodine value = 0.

$C_{14}H_{28}O_2$ requires equivalent wt. 228.

Fraction (2). Wt. 1·98.

0·1506 g. neutralised 6·14 cc. *N*/10 NaOH. Equivalent wt. = 245·3.

0·1322 g. absorbed 0·5 mg. I_2. Iodine value = 0.

$C_{15}H_{30}O_2$ requires equivalent wt. 242.

0·16 g. of this acid dissolved in 10 cc. pure ethyl alcohol showed no optical activity.

The first fraction of these acids remained quite liquid when kept at a temperature of 0° for several days. The viscosity was rather higher than at room temperature but there was no sign of crystallisation. The higher boiling fraction on cooling to 0° deposited white, waxy crystals but the acids as a whole did not solidify. It seems that one acid crystallised out whilst another remained liquid. This fact, together with the knowledge that no C_{15}-acid is definitely known to be present in nature, suggests that the second fraction is probably a mixture of almost equal quantities of C_{14}- and C_{16}-acids. The C_{16}-acid would presumably be just solid at room temperatures.

D. *Oxidation of the fatty acids from the cat's kidney.*

The oxidation was carried out by two methods:

(1) *Hilditch's* [1926] *method.* Oxidation of the mixed acids by means of hydrogen peroxide (90/100 vols.) in glacial acetic acid at 70° as described by Hilditch gave rise to a dihydroxystearic acid, M.P. 92°. No sativic acid fraction was obtained.

(2) *Dilute alkaline potassium permanganate.* The mixed acids (iodine value 48) were also oxidised by dilute, ice-cold potassium permanganate in alkaline solution as was done by Hartley [1907] with acids from the liver.

Acids soluble in light petroleum formed 48 % of the original mixed acids. The iodine value was 27·3.

An acid insoluble in light petroleum, soluble in ether was obtained. This had M.P. 131° and was therefore dihydroxystearic acid.

An acid insoluble in light petroleum and ether, but soluble in boiling water, from which it crystallised on cooling, was also found. It had M.P. 164–166° corresponding with tetrahydroxystearic acid.

These two acids are derived from oleic and linoleic acids respectively. The presence of the latter acids in the fatty acids from the cat's kidney is therefore demonstrated.

E. *Bromination of the acids from the cat's kidney.*

1·38 g. of the liquid acid fraction (iodine value 67·5) were dissolved in 30 cc. of ether, and a 1 % solution of bromine in ether was added drop by drop to the ice-cold solution until excess of bromine was present. The solution was allowed to stand overnight in ice. A small white deposit was obtained which was filtered off through a weighed Jena glass crucible.

The ether was removed from the filtrate and the residue after weighing was treated with light petroleum (B.P. 60–80°). A dark brown precipitate was obtained; this was likewise filtered off and weighed.

1·38 g. of mixed liquid acids gave 1·85 g. bromination products, 0·02 g. insoluble in ether, 0·11 g. insoluble in light petroleum (B.P. 60–80°) and 1·72 g. of material (by difference) soluble in light petroleum.

Rollett [1909] has shown that about half of the linoleic tetrabromides produced from ordinary linoleic acid are soluble in light petroleum. Allowing for this, about 0·25 g. of the bromides out of the 1·85 g. are tetra- or polybromides. When it is considered that about 60 % of the weight of a tetrabromide is bromine, it is obvious that the amount of acids more unsaturated than oleic acid in the 1·38 g. of mixed acids must be very small. It is safe to conclude therefore that nearly all the iodine absorption of the liquid acid fraction is due to the presence therein of oleic acid.

The lipoid fractions of cat's kidney.

A number of cat's kidneys were first exhaustively extracted with acetone and the residue was then extracted with ether. The two lipoid fractions obtained correspond approximately with the glycerides and the phospholipins respectively. For comparative purposes a similar treatment was given to a quantity of beef kidney and the results of the investigations on these fractions are summarised in Tables III–V.

Table III. *Appearance of extracts.*

Fractions	Cat	Beef
(1) Acetone extract	Deep orange viscous oil	Deep orange wax
(2) Ether extract	Pale orange semi-solid	Pale orange waxy extract
1*a*. Fatty acids from saponification of Fraction (1)	Liquid	Solid
2*a*. Same from Fraction (2)	Waxy solid	Solid

Table IV. *Distribution of lipins.*

Fraction	Cat kidney (1) %	Cat kidney (2) %	Beef kidney %
Acetone extract	3·01	1·85	0·87
Ether extract	1·99	2·00	1·26

Table V. *Iodine values.*

Fraction	Cat kidney (1) %	Cat kidney (2) %	Beef kidney %
Acetone extract:			
Before saponification	55·0	69·3	117·9
Fatty acids from saponification	56·7	63·9	130·2
Ether extract:			
Before saponification	67·2	59·8	84·0
Fatty acids from saponification	74·9	55·5	95·2

Discussion.

Mottram [1916] considered that the abnormally low iodine value of the cat kidney fat was due to a process of fatty infiltration of the kidney. This view was based on the fact that the cat kidneys contained, on the average, about 5 % of fat compared with the more usual value of 2–3 % for other animals, and that sections of the kidney stained for fat showed an abnormally large number of fat droplets lining the tubules. Assuming that 3 % of the

fats had the customary iodine value for organ fat of 135, which is true for most animals, then the additional 2 % represents infiltrated fat. If the infiltrating fat is adipose tissue fat, and it is to be presumed that Mottram intended this, then the iodine value of the mixed acids would be theoretically 107. This is greatly in excess of the observed iodine value of approximately 60 for the fatty acids from the cat's kidney. The infiltration of adipose tissue fat of iodine value 65 could obviously never account for an iodine value of the mixed acids lower than this value, yet in practice values as low as 50 are observed. It is found experimentally that the iodine value of adipose tissue fatty acids from cats is always about 65. For Mottram's view to be correct, therefore, it must mean that the iodine value of the fatty acids of the "élément constant" for the cat's kidney must be considerably below the accepted normal value of 135. In any case an infiltration hypothesis would not account for the remarkable fluidity of the acids from the cat's kidney. Adipose tissue fatty acids at room temperatures are quite solid, whereas the renal fatty acids of the same or even lower iodine value, are semi-liquid. There must be some fundamental difference in the nature of the fat in the kidney of the cat compared with that of other animals, for it is impossible by making use of values which are true for the latter to account at once for the lower iodine value and the more liquid nature of the fatty acids obtained from the cat's kidney. The presence of some fatty acids which possess a very low or zero iodine value and a high fluidity, for example liquid saturated acids, would reconcile these observations. The experimental procedure was, in consequence, directed towards the isolation of such acids if these were present. The actual isolation of completely saturated fatty acids which are liquid at room temperature confirms the truth of the above suggestion. The very limited amount of these acids at present available makes it impossible to define their constitutions. It is hoped however to proceed with the investigation of this problem when sufficient quantity of the material has been collected; in this preliminary investigation the loss of material has been very heavy, but for the future preparations it is believed that the acids will be isolated in greater yield by a much simpler process.

So far as is known the only naturally occurring liquid saturated fatty acids with more than fourteen carbon atoms are the tuberculostearic and phthioic acids found by Anderson [1929] and Anderson and Chargaff [1930] among the fatty acids from tubercle bacilli. These acids are isomeric with stearic and cerotic acids respectively. Branching of the chain may be a possible explanation of the liquid nature of these acids, since for example α-amyl-n-nonoic acid is still liquid at $-10°$ and β-butyl-n-decoic acid has M.P. 4°.

The experimental evidence suggests that these liquid saturated acids are present in both the simple glyceride and phospholipin fractions of the fats in the cat's kidney. Both these fractions are noticeably more liquid than the corresponding fractions from beef kidney although the iodine values of the latter are much higher. The differences in iodine value are more marked in

the simple glyceride fraction, the values from the beef kidney being almost double those from the cat's kidney. On saponification, beef kidney gives rise to solid acids from both the simple glyceride and phospholipin fractions. The glyceride fraction from cat's kidneys gives rise on saponification to acids which are mainly liquid, whilst the phospholipins yield a waxy product. The inference is that it is in the simple glycerides that the larger proportion of liquid saturated acids is present. The solid saturated acids are apparently present to a greater extent in the phospholipins.

The occurrence of this type of liquid saturated fatty acids in the cat's kidney raises questions of no little physiological interest, but a discussion of these may more appropriately be reserved for a future communication.

Summary.

The percentage amounts and iodine values of the fatty acids from the kidneys of 20 cats have been determined. The mixed fatty acids obtained by saponification of the kidney fats have been examined. They are shown to consist of palmitic, stearic, oleic and linoleic acids, together with at least two new liquid saturated acids. The constitutions of the latter have not yet been ascertained. A comparison has also been made between the simple glyceride and phospholipin fractions obtained by the same processes from cat and beef kidneys.

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IX. THE PERMEABILITY OF HUMAN EPIDERMIS TO ULTRA-VIOLET IRRADIATION.

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The work of Rosenheim and Webster [1927] and Windaus and Hess [1927] has shown that the irradiation of ergosterol with ultra-violet rays produces vitamin D. Since Hess, Weinstock and Helman [1925] found irradiated lanolin to be antirachitic, it may be concluded that ergosterol or a similar sterol is associated with cholesterol in the sebaceous material of the skin.

It has been shown by Hume, Lucas and Smith [1927] that ordinary impure cholesterol after irradiation, if gently rubbed into the intact shaven skin, can protect rabbits on a rachitogenic diet from rickets. The cholesterol was dissolved in ether and the solution poured on to a glass plate, and irradiated under a mercury vapour quartz lamp. The irradiated cholesterol was mixed with cottonseed oil to facilitate inunction, and rubbed into shaved areas on the backs of rabbits, these areas being covered with wash-leather jackets to prevent ingestion by the mouth.

Absorption through the skin of vitamin D, produced in skin fat, may not however be the only, or the usual, way in which the antirachitic effect of ultra-violet light is communicated to the animal body. Rosenheim and Webster have shown that only 0·0001 mg. of irradiated ergosterol daily is required to protect a rat from rickets or even to cure it. Moreover the amount of ultra-violet irradiation which reaches the surface of the rat is very small, since, with the exception of the ears, feet and horny tail, the rest of the rat is covered with fur; yet it is enough to protect the rat from rickets. It may be that enough ultra-violet rays of sufficiently short wave-length to activate the ergosterol may penetrate the epidermis and reach the ergosterol contained in the capillaries lying just below. The epidermis on the parts of the human body other than the palms of the hands and soles of the feet, is approximately 50μ thick at the peaks of the dermis where the network of capillaries is thickest.

Previous observations.

Our knowledge of the permeability of skin and epidermis to ultra-violet light of different wave-lengths largely depends on the observations of Hasselbalch [1911] made 20 years ago, using light of wave-lengths ranging between $437\mu\mu$ and $289\mu\mu$. His conclusions are those commonly referred to in the

literature. Hasselbalch's method was to use skin from a corpse, placing it between two discs of optically flat clear quartz so fitted into a short brass cylinder as to form an air-tight chamber in which the skin was pressed flat between the discs. He used a Kromayer quartz mercury vapour arc lamp and a quartz spectrograph. The skin was placed 5 cm. in front of the slit of the spectrograph which was widely open. To receive the photographic image he used Solio white matt printing-out paper. The time necessary to produce a suitable image through the skin was recorded. The dark-slide was then lowered by the rack and pinion and the illumination repeated through the quartz discs only, the image being received immediately above the preceding one and the exposure being continued until the two images of a selected mercury vapour band were of equal density. Owing to the use of Solio paper it was possible, without spoiling the image produced, to interrupt the exposure from time to time, to examine both photographs and stop the exposure at the desired point. When two images of equal density of one of the mercury-vapour bands of longer wave-length had been obtained and the respective times recorded, the same piece of paper could be replaced and the exposures continued for bands of shorter wave-length.

In the calculations which were the outcome of his experiments, Hasselbalch made use of absorption coefficients, calculating these according to the formula

$$\frac{J}{J_0} = 10^{-\epsilon' d} \quad \text{or} \quad \frac{-\log \frac{J}{J_0}}{d} = \epsilon'$$

where ϵ' is the coefficient of absorption, J is intensity of the transmitted light, J_0 that of the incident light, and d is the thickness of the skin in millimetres. The modern practice is to use centimetres, but Hasselbalch used millimetres and to render comparison easier his practice has been followed in presenting my results. The values for the transmitted and incident light are inversely proportional to the times of exposure required to produce an image of the same density. Thus

$$\frac{J}{J_0} = \frac{t'}{t}$$

where t and t' are the times required to produce images of the same density from transmitted and incident light respectively.

The absorption coefficients for skin, calculated by Hasselbalch, varied from 2·3 for a wave-length of 437 $\mu\mu$ to 5·2 for a wave-length of 313 $\mu\mu$ which was the shortest he was able to examine. For epidermis his figures were:

Wave-length	404	360	334	313	302	294	289 $\mu\mu$
ϵ'	2·3	4·2	6·6	8·5	12·4	17·5	39

Anderson and Macht [1928, 1, 2] made two series of experiments on the penetration of ultra-violet rays through living skin. In their first series they irradiated the skin of a living anaesthetised rabbit, reflected from the body, with monochromatic light from a mercury vapour arc lamp. They recorded

the energy transmitted through the skin by means of a thermopile and galvanometer. The result showed that between the wave-lengths 360 $\mu\mu$ and 280 $\mu\mu$ the percentage transmission increased from 11·4 % to 56·3 % and that there was then a drop to 23 % at 265 $\mu\mu$ with a further rise to 42·8 % at 253 $\mu\mu$.

This paper was criticised by Hill [1928] and by Bachem [1928]. Hill's criticism is chiefly based on the results of his own physiological experiments. He showed the great protection against ultra-violet irradiation afforded by a layer of omentum which is far thinner than skin, and also the protection to tubercle bacilli afforded by a thin layer of serum. Bachem [1928] drew attention to the possible influence of stray light rays of other wave-lengths due to the imperfect production of monochromatic light, and to the error due to the fluorescence of the skin. These fluorescent waves affected the thermopile, and the energy thereof was registered as energy from ultra-violet waves coming through the monochromator.

Anderson and Macht in their second paper [1928, 2] used a special photometer to control the thermopile method and found that the amount of stray light was negligible. They admitted the error due to the fluorescence of the skin and corrected it. Nevertheless their findings show a rise of transmission from 7·5 % to 9·8 % between the wave-lengths 302 $\mu\mu$ and 280 $\mu\mu$. This is contrary to the findings of workers on the ultra-violet absorption of other protein substances.

It is not out of place at this juncture to point out that the error due to the inclusion of fluorescent rays can almost certainly be excluded when the transmitted rays are analysed by the spectroscopic method instead of a thermopile. In this method any fluorescent light from the skin is recorded on the photographic plate as visible light and not as ultra-violet. A source of error would only be possible if a proportion of the incident light were converted into light of a shorter wave-length by fluorescence. This form of conversion has not yet been established.

Methods used in the present work.

Two years ago I repeated Hasselbalch's experiments with human skin, but using parallel light. The results obtained, expressed as absorption coefficients per millimetre, indicated that the skin used was 100 to 10,000 (according to the wave-length) times more opaque than that used by him. In searching for a solution of this discrepancy the conclusion was reached that it was due to the loss of intensity of the issuing light caused by scattering, owing to the different conditions of our experiments.

Skin is not optically homogeneous. Incident light, in addition to being diffusely reflected at the surface, is irregularly refracted as it passes through the layers of cells which compose it. When therefore skin is interposed between the light-source and the slit of the spectrograph, many of the rays are reflected and the remainder so much bent that they miss the slit altogether, while others pass through at such an angle that they impinge on the wall of the collimator

tube and not on the prism. The result is that only a fraction of the incident light which is unabsorbed by the skin is recorded and measured against the incident light. If the incident rays are parallel the effect of scattering is maximal, and this is the reason why my early figures are higher than those of Hasselbalch, who apparently did not use parallel light.

When light impinges on the skin of a living animal scattering is not of such great importance, because the transmitted light does not subsequently encounter a narrow slit. Although much diffuse reflection occurs at the surface, rays of light striking the skin surface at one point ultimately reach the blood vessels, though possibly at positions widely separated from the point of incidence.

Another explanation of the exaggerated value for the absorption of skin obtained in experiments of this type is that the light traverses a devious path, the length of which is not necessarily represented by the thickness of the skin, which is the measure used for the calculation of absorption coefficients. It is not therefore possible to determine the absorption coefficient, which depends for its estimation on the thickness of the layer actually traversed. For this reason the expression of the absorption of light by the skin by means of absorption coefficients has been abandoned in these experiments, and the degree of absorption for light of different wave-lengths is expressed in terms of relation between the logarithms of incident and transmitted light respectively, $\left(\log \frac{J_0}{J}\right)$, as measured by the respective times of exposure needed to obtain images of equal density after passage through the particular thickness of skin or epidermis used. As was stated previously the times of exposure are inversely proportional to the respective intensities of the light,

$$\left(\log \frac{J}{J_0} = \log \frac{t'}{t}\right).$$

In order to minimise the effect of scattering of light, it was decided to insert scattering surfaces in front of the light-source in the form of ground quartz discs, through which the light would pass in tests both with, and without, the skin. The skin was placed between two clear quartz discs as before (Fig. 1, 1 and 1′). One of these was recessed into an outer casing (2), with a fine screw thread on the outer side, while the other disc was similarly recessed into a thick ring of metal (3), which slid into (2), and fitted sufficiently well to make the cavity air-tight. The whole of this was then screwed into an outer casing (4), with a thread on the inner side. The scattering of the incident light was produced by two quartz discs each ground on both surfaces (5 and 5′) fixed with plasticene on to the inner and outer surfaces of the front surface of (4). The whole then slid into the hood of the spectroscope from which the front cap had been unscrewed.

The light-source was a Hewittic quartz mercury vapour lamp working at 2·25 amperes. It was enclosed in a metal case and the light emerged through an aperture $1\frac{1}{2}$ inches in diameter.

The introduction of the ground quartz discs reduced the intensity of the light incident on the slit to such a degree that considerably longer exposures were needed. For this purpose a photographic shutter was made of a piece of flat metal blackened on both sides attached to the long arm of a lever, the short arm of which was operated by the armature of an electromagnet. This was placed in front of the cell holding the epidermis. When an electric current passed through the magnet the armature was pulled down, and the shutter on the long arm raised, thus making the exposure. The duration of the exposure depended on the length of time the current flowed. The timing apparatus consisted of a wooden pulley geared to a gramophone motor in a ratio

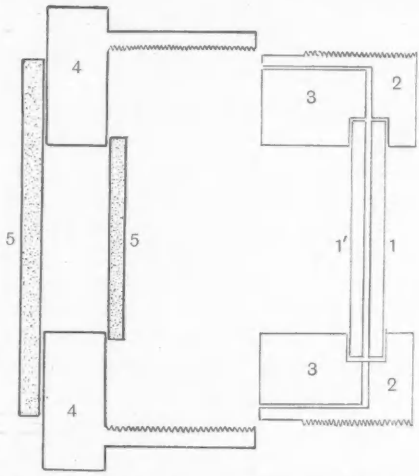

Fig. 1. Air-tight cell arranged to hold epidermis.

which produced one revolution in 5 seconds. A series of wooden discs into each of which was recessed a brass sector was attached to the face of the pulley. Two strips of springy brass were fixed so as to wipe against the face of the disc on the pulley and complete the circuit during the time they passed over the brass sector. The ends of the strips were in the line of the radius of the pulley face, and therefore reached the edge of the brass sector simultaneously. The shutter by its own weight fell back in front of the cell. Sectors were made to give exposures of from 1/10 sec. to 3 sec., varying by 1/10 sec. Instead of Solio paper, used by Hasselbalch, Ilford "Process" plates (H & D = 50) were employed.

The constancy of the source of illumination was examined by taking a series of spectrographs with the same period of exposure, one below the other, on the same plate. It was found that after the lamp had been lit for 30 minutes the light was so steady that there was no discernible difference of intensity between any of the spectrographs.

With a piece of human skin in position a series of exposures was made on a plate, varying between 1 second for the long wave-lengths and 30 minutes for the short ones. These provided the measure of the light transmitted through the skin or epidermis, which was then removed. With the cell otherwise the same, shorter exposures were made on further plates by means of the electromagnet shutter. These gave the measure of the incident light.

The two series of plates were developed for an equal time with developer at the same temperature. Then for each wave-length the intensity of a line in the series obtained from the transmitted light was matched with that of the same line in the series obtained from the incident light. The times of exposure of the two were then noted. If no two images showed exact equality of intensity, the average was taken between the two exposures of incident light, which gave images of the selected line of intensity respectively greater and less than the intensity of that from the transmitted light.

A trial of this apparatus showed that the diminution of the intensity of the incident light by scattering at the ground quartz surface was so great that no image of any line of shorter wave-length than 313 $\mu\mu$ could be obtained through skin of 0·2 mm. thickness except by exposures of 24 hours and longer.

Skin was therefore abandoned in favour of epidermis, a change the more justifiable, because it is only the thin covering of epidermis (of thickness about 0·05 mm.) which in the living body is interposed between the dermal capillaries and the source of illumination. Epidermis was obtained from the front of the fore-arm by means of a blister produced with chloroform vapour. Transverse sections, appropriately stained, showed that the stratum granulosum was included in the separated portion. These pieces of epidermis were stored in a refrigerator in tap-water, and when placed in the cell were moistened with tap-water.

Experiments with human epidermis.

1. *Diminution of error due to scattering of light by interposing ground quartz discs.*

An experiment was made with a specimen of human epidermis 0·08 mm. thick and values for $\log \frac{J_0}{J}$ were obtained for wave-lengths corresponding to the chief lines in the violet and ultra-violet parts of the emission spectrum of the mercury vapour arc, *i.e.* from wave-length 437 $\mu\mu$ to 256 $\mu\mu$. In the first experiment (*a*) the incident light was previously scattered by the insertion of ground quartz discs as described above. A second experiment (*b*) was then performed with the light made approximately parallel. In order that images of submaximal intensity should be obtained for long wave-lengths of the incident light, the lamp was placed at a distance of 11 feet from the spectrograph. The resulting figures are shown in Table I, cols. *a* and *b*, and are reproduced graphically in Fig. 2, *a* and *b*, where the ordinates are values of $\log \frac{J_0}{J}$ and the abscissae are wave-lengths expressed in millimicrons.

Table I. *Absorption of violet and ultra-violet radiations by human epidermis 0·08 mm. thick.*

Wave-length in $\mu\mu$	Values of log J_0/J (*a*) Using light scattered by ground quartz discs	(*b*) Using parallel light
437	0·55	1·68
404	0·62	1·82
360	0·76	2·14
334	1·02	2·52
313	1·14	2·93
302	1·40	3·28
294	1·66	3·55
289	2·44	—
264	2·80	—
256	2·66	—

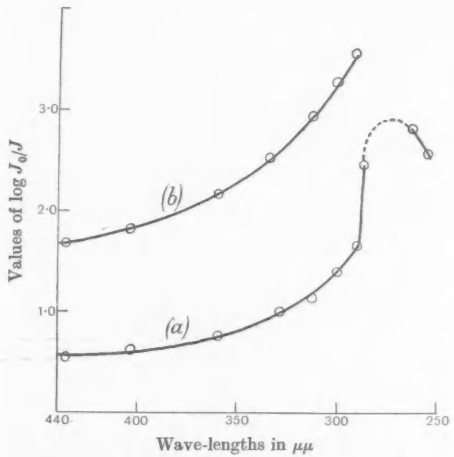

Fig. 2. Effect of using incident light scattered by ground quartz surfaces. Absorption curves of human epidermis (0·08 mm. thick).
a. Incident light scattered. *b*. Incident light parallel.

The apparent increase in the transmission through the epidermis when the incident light was previously scattered was unexpectedly great, being from 13 (for wave-length 437 $\mu\mu$) to 78 (for wave-length 294 $\mu\mu$) times greater than in Exp. *b*, where parallel light was used. As the same piece of epidermis was used in both cases, this difference is only an apparent one, due not to the epidermis, but to the method of illumination, the only thing changed in the two experiments. For wave-lengths less than 294 $\mu\mu$ the scattering by the epidermis becomes so great, when parallel light is used, that the light entering the slit is insufficient to produce an image with the exposure used, and the rest of the graph is unobtainable.

Using the ground quartz discs experiments were made with specimens of

epidermis from two other people. These specimens (Fig. 3, *a* and *b*) were thinner than those used in the above experiment being 0·065 mm. and 0·05 mm. in thickness respectively. The values for $\log \frac{J_0}{J}$ were therefore lower and readings were obtained for radiations of the wave-length 246 $\mu\mu$ with both specimens and for wave-length 240 $\mu\mu$ for one (Fig. 3 *b*). These

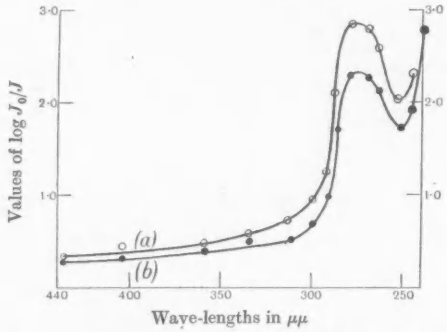

Fig. 3. Absorption curves of epidermis from two different individuals, using scattered incident light.
a. Thickness 0·065 mm. *b*. Thickness 0·05 mm.

graphs resemble in shape the absorption curves of the amino-acids tryptophan and tyrosine, shown in Fig. 7 [Ward, 1923], of serum-albumin [Smith, 1929] and of caseinogen determined by myself (see Fig. 5).

Tyrosine has been found in epidermis by Buchtala [1912] and tryptophan, though not actually shown to be present in epidermis, has been found in epidermal structures such as wool to the extent of 1·8 to 1·87 % [Marston, 1928].

2. *Diminution of error due to scattering of light by employing clarified specimens of epidermis.*

So far, it has been shown that the apparent opacity of epidermis to ultra-violet light may be diminished nearly a hundredfold by altering the optical conditions of the experiments, but even the method of employing previously scattered light by no means eliminates the disturbing effects of subsequent scattering in the epidermis. Recourse was therefore had to the method of clarification, by immersing the epidermis in some liquid the refractive index of which approached that of the materials of which epidermis is composed.

The choice of clarifying agents was governed by two conditions. The reagents should not alter the chemical composition of the epidermis, nor have of themselves an absorption of ultra-violet light in the region under investigation. For this reason the ordinary clarifying agents of microscopy, xylene, toluene, chloroform, etc., were unsuitable. The reagents chosen were glycerol and glacial acetic acid, both of which fulfilled the necessary conditions. Of

these glacial acetic acid was the better, judging by visible standards. The following experiments showed that these reagents did not permanently change the chemical constitution of the epidermis.

A piece of the same epidermis as was used for the experiment shown in Fig. 2 was placed in tap-water and a photograph taken of the transmitted spectrum using parallel light. The results are shown in Table II, col. 1 and

Table II.

Wave-length in $\mu\mu$	Values of $\log J_0/J$						Per-centage of incident light trans-mitted in Exp. 4	Percentage of incident light transmitted according to Hasselbalch	
	1	2	3	2′	3′	4		Epidermis 0·04 mm. thick	Calcu-lated for epidermis 0·08 mm. thick
437	1·78	0·79	1·70	0·40	1·82	0·02	96	—	—
404	1·95	0·80	1·87	0·40	1·94	0·04	91	81	65
360	2·23	1·00	2·08	0·52	2·18	0·10	81	68	46
334	2·75	1·20	2·57	0·60	2·62	0·19	65	54	29
313	3·10	1·34	3·00	0·71	2·90	0·26	55	46	21
300	3·52	1·61	3·24	0·84	3·19	0·35	44	32	10
294	3·86	2·03	3·54	0·94	3·40	0·58	26	20	4
289	—	3·12	—	1·40	—	1·54	2·9	3	0·09
280	—	3·60	—	1·78	—	1·78	1·7	—	—
270	—	3·05	—	1·81	—	1·83	2·0	—	—
265	—	3·13	—	1·61	—	1·60	2·6	—	—
254	—	3·33	—	1·56	—	1·34	4·6	—	—
246	—	—	—	1·72	—	1·43	1·5	—	—
240	—	—	—	2·71	—	—	—	—	—
238	—	—	—	3·16	—	—	—	—	—

(1) Epidermis, thickness 0·08 mm. in tap-water, light parallel.
(2) " " cleared in glycerol, light parallel.
(3) " " washed and replaced in tap-water, light parallel.
(2′) " " cleared in glacial acetic acid, light parallel.
(3′) " " washed and replaced in tap-water, light parallel.
(4) " " cleared in acetic acid, incident light scattered with ground quartz discs.

in graphic form in Fig. 4, curve 1. The epidermis was then placed in glycerol for about an hour after which no further clearing could be observed. It was then clamped between the quartz discs and a similar series of photographs taken. The values of $\log \frac{J_0}{J}$ are shown in Table II, col. 2 and in graph form in Fig. 4, curve 2. The glycerol was then washed out and the epidermis replaced in tap-water. The series of photographs obtained with this material provided measurements of $\log \frac{J_0}{J}$ reproduced in Table II, col. 3 and in Fig. 4, curve 3, which coincides almost exactly with curve 1. The epidermis when cleared with acetic acid furnished data to produce the figures of Table II, col. 2′ and the curve 2′, Fig. 4. Once more it was replaced in tap-water and from the figures obtained in this experiment, the curve 3′ Fig. 4, was set out and found to be almost identical with curves 1 and 3, showing that the clearing reagents did not permanently alter the constitution of the epidermis.

Finally the epidermis was placed in ether to remove any possible fat and

then cleared in acetic acid, and a series of photographs were taken with the ground quartz discs in position, with the idea of still further reducing any possible scattering. The values for $\log \frac{J_0}{J}$ obtained in this experiment are shown in Table II, col. 4, and the corresponding curve 4 in Fig. 4. In accordance with the naked eye appearance acetic acid proved a better clarifying agent than glycerol for ultra-violet light, for the less degree of absorption measured is due no doubt to a less degree of scattering.

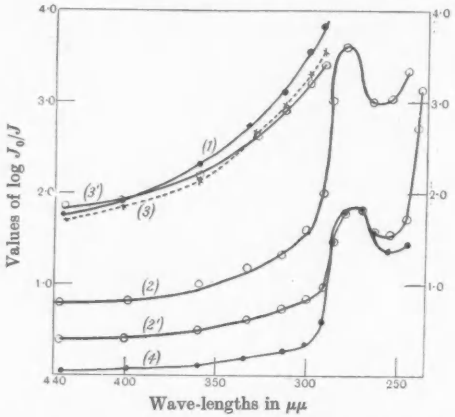

Fig. 4. Absorption curve from epidermis of the same individual as in Fig. 2. Thickness 0·08 mm.

Curve	1.	Epidermis in	tap-water. Light parallel.
,,	2.	,,	glycerol. Light parallel.
,,	3.	,,	tap-water. Light parallel.
,,	2′.	,,	glacial acetic acid. Light parallel.
,,	3′.	,,	tap-water. Light parallel.
,,	4.	,,	glacial acetic acid. Light scattered.

In comparing in Fig. 4 the curves 2, 2′ and 4, the first thing noticed is that in general shape, the curves are similar to each other, to those in Fig. 3 and to curve *a* in Fig. 2. The actual values of $\log \frac{J_0}{J}$ are not comparable in the different experiments, as the specimen of epidermis used for the tests illustrated in Fig. 4 was thicker than those used for the experiments shown in Fig. 3.

Curve 4 (Fig. 4) showed a very interesting phenomenon. The introduction of ground quartz discs reduced the apparent opacity of epidermis, even when partially cleared, to a marked extent, as far as the wave-length 294 $\mu\mu$. After that the curve coincided very closely with curve 2′, corresponding to epidermis cleared similarly with acetic acid, but exposed to parallel incident light. This would show that for light of wave-length 294 $\mu\mu$ and shorter, the effect of true absorption is so great as to dwarf the effect due to scattering. It is probable therefore that curve 4 shows the best approximation to the true absorption of ultra-violet light by human epidermis.

With the idea of examining in detail that part of the curve in which there is true absorption, a piece of the same epidermis was photographed in water, glycerol and acetic acid in turn. In order to obtain records of the shortest wave-lengths possible, it was decided to use, in addition to the clarifying reagents and the ground quartz discs, a quartz lens placed so as to render the rays, which had previously been allowed to come haphazard from the lamp, parallel before reaching the first scattering surface of the first quartz disc. The intensity of illumination being thereby increased, the time of exposure necessary to obtain records from the shorter wave-lengths was diminished. From these observations values for $\log \frac{J_0}{J}$ were obtained, and curves were drawn to show absorption for lines of the mercury vapour spectrum corresponding to short wave-lengths hitherto unmeasured from lack of intensity of the transmitted light. This gave greater accuracy to the curves.

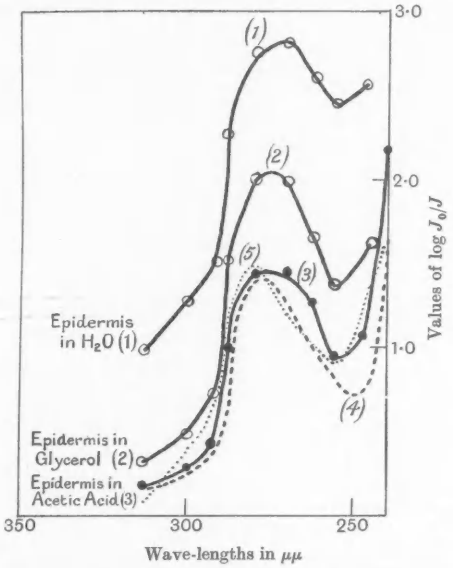

Fig. 5. Repetition of experiment on epidermis from the same individual as that used for experiments illustrated in Figs. 2 and 4, but with incident light collected on to the ground quartz discs. Thickness of epidermis 0·08 mm.

Continuous Curve 1. Epidermis in water.
" 2. " glycerol.
" 3. " acetic acid.

For comparison:
Dotted Curve 4. 2·5 % serum-albumin. Thickness of layer 1 mm.
" 5. 2 % caseinogen. Thickness of layer 1 mm.

The curve of absorption by epidermis cleared with glacial acetic acid (Fig. 5, curve 3) showed great similarity to the absorption curve of a 2·5 %

solution of serum-albumin or 2 % caseinogen solution, except that for serum-albumin the drop in the region of wave-lengths 270 $\mu\mu$ to 250 $\mu\mu$ is not so great.

Damp epidermis freed from adherent moisture by pressing between filter-paper contains 40·4 % of water, and the absorption curve illustrated in Fig. 5 is not unlike that of a similar thickness of a 2 % solution of caseinogen both qualitatively and quantitatively. The curves shown in Fig. 7 indicate that the

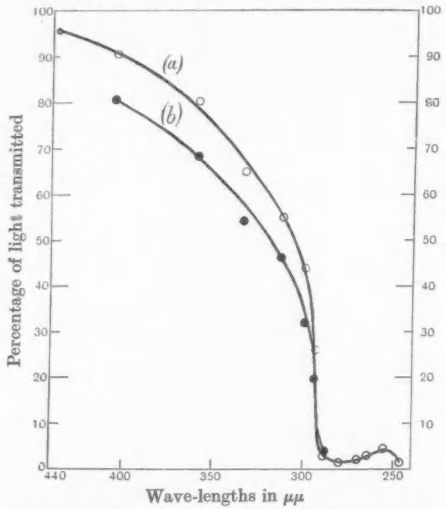

Fig. 6. Curves showing percentage transmission of light through epidermis:
a. Curve derived from Exp. 4. Epidermis 0·08 mm. thick.
b. Curve derived from Hasselbalch's experiment with epidermis 0·04 mm. thick.

opacity of epidermis to ultra-violet rays between the wave-lengths 313 $\mu\mu$ and 240 $\mu\mu$ would appear to be principally due to the content of tyrosine and tryptophan, and to be of the same order as that of the proteins, albumin and caseinogen. Cystine does not appear to be responsible for any material part of the opacity of epidermis to ultra-violet rays in the region under consideration, because the great rise in its absorption does not begin until the wave-length 225 $\mu\mu$.

The best approximation to the true absorption of ultra-violet light by epidermis would appear to have been obtained in Exp. 4 (Table II; curve 4, Fig. 4). Translating the logarithmic figures into arithmetical numbers the percentage of incident light which penetrates and is transmitted through the epidermis and reaches the blood-stream is shown in Fig. 6, curve (*a*) and in the last column but one of Table II.

It appears then that in the range of wave-lengths of the sun's light arriving at the earth's surface which are active in the cure of rickets amounts varying from 55 % for wave-length 313 $\mu\mu$ to 26 % for wave-length 294 $\mu\mu$ may be transmitted to the dermal capillaries.

For comparison a curve illustrating Hasselbalch's experiment with epidermis is included in Fig. 6 (curve *b*). The epidermis which he used was 0·04 mm. thick, whereas the epidermis which provided the material in the present work was 0·08 mm. thick. Nevertheless with the much thicker epidermis the proportion of transmitted light recorded for wave-lengths 404 $\mu\mu$ to 289 $\mu\mu$ was greater than that estimated by Hasselbalch. On the basis of his figures the proportion of light transmitted through epidermis of 0·08 mm.

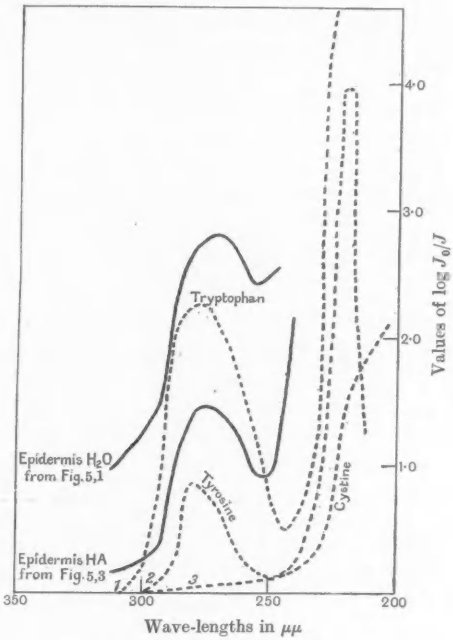

Fig. 7. Absorption curves.
1. Epidermis in water } from Fig. 5.
2. ,, acetic acid }
3. Tryptophan.
4. Tyrosine.
5. Cystine.

thickness has been calculated (see Table II last column). These figures compared with those obtained in the present work show human epidermis to have from 1·5 to 30 times the transparency found by him for radiations of wave-lengths 404 $\mu\mu$ to 289 $\mu\mu$ respectively. The reason for this discrepancy is probably due to a failure on his part to differentiate between the loss of transmitted light by scattering, which loss is progressively greater as the wave-length decreases, and the loss by true absorption. It must be emphasised that much more is lost to the spectrograph by scattering when skin is irradiated

under the condition, of these experiments, than would be lost to the animal during irradiation.

SUMMARY.

1. The apparent absorption of ultra-violet light by epidermis as determined by the usual methods of photometry is not entirely due to true absorption, but is in part due to scattering of the incident light by the epidermis, which is not optically homogeneous.

2. This scattering while causing great diminution of the intensity of the light received by the spectrograph causes much less interference in transmission of the light to the underlying tissues when skin is irradiated *in vivo*.

3. For light of wave-length $300\mu\mu$ and less the effect of scattering is dwarfed by true absorption by the epidermis.

4. In shape these absorption curves resemble those of many proteins and amino-acids, *e.g.* serum-albumin, caseinogen, tryptophan and tyrosine.

5. The percentage of ultra-violet light of physiologically active wave-lengths transmitted through the epidermis is higher than previously recorded by Hasselbalch, and is calculated to be about 1·5 to 30 times greater for wave-lengths from $404\mu\mu$ to $289\mu\mu$ respectively.

My thanks are due to the Lister Institute of Preventive Medicine for facilities in carrying out these experiments, and to Sir Charles Martin for his great help, without which the work could never have been completed.

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X. THE RELATION BETWEEN CYSTINE YIELD AND TOTAL SULPHUR IN KEMP AND OUTER-COAT ANIMAL FIBRES.

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In previous publications [Rimington, 1929, 1 and 2] it has been shown that practically the entire sulphur of purified wool and animal hair can be accounted for as cystine, the latter being determined colorimetrically and its quantity compared with the total sulphur of an aliquot portion of the hydrolysate. Several different varieties of wool and of hair were examined with identical results. It was noted, however, in the case of some animal hairs, more particularly of a sample of camel hair, examined, that the agreement between sulphur and cystine values was less close than in the case of true wool fibres. Such a difference might possibly be explained on the ground that the medullary cells of the hair shaft contain sulphur compounds other than cystine. Medulla is present in most animal hairs; it is absent however from true wool fibres.

In order to test this possibility and to complete the series of observations already reported, it was decided that an examination should be made of some typical kemp, of lambs' birth-coat, which is also medullated, and of further samples of camel hair. The investigation was rendered difficult by the necessity of sorting each sample by hand; in some cases it took several weeks to pick out sufficient material for analysis. In the case of camel hair, and Scotch blackface wool, one is also confronted by the difficulty of classification, nearly all gradations being met with between true wool and true kemp.

Experimental.

Each sample, after separation, was degreased in warm benzene and washed in the manner already described [Rimington, 1929, 1]. Samples of 1 g. were taken for hydrolysis. These were boiled for 5 hours with 20 cc. of 20 % hydrochloric acid. Two portions of 5 cc. each were then withdrawn for determination of total sulphur content, which was carried out by Rimington's [1930, 1] modification of the Benedict-Denis method for the determination of sulphur in urine. Another 5 cc. sample was measured into a 50 cc. graduated flask, diluted somewhat and brought to about $N/10$ acidity by cautious addition of 20 % sodium hydroxide solution. After filling to

50 cc. and filtering, this solution (2 cc.) was employed for the colorimetric determination of cystine, Folin and Marenzi's method as amended by Rimington [1930, 2] being employed.

Since, by Folin and Marenzi's [1929] technique it is now possible to obtain a reagent free from molybdenum compounds and giving no colour with pure tyrosine, this method is to be preferred to that of Sullivan [1926], which is technically somewhat difficult and which gives only a limited proportionality between colour intensity and cystine concentration. In the present study it was not considered necessary to employ Sullivan's method at all since earlier work [Rimington, 1929, 1] had shown the absence from hirsute structures of disulphide linkages other than those due to cystine. Where determinations were made of the total sulphur of the dry material, these were carried out as described in previous publications.

The results are recorded in Table I.

Table I. *Hydrolysis of 1 g. material by 20 cc. of 20 % HCl for 5 hours.*

Material	Total S % dry wt.	Cystine in hydrolysate by Folin-Marenzi-Rimington method g.	Total S of hydrolysate (as cystine) g.	Ratio*
Camel hair (kemp-free) I	3·25	0·1100	0·1169	+5·90
,, kemp I	—	0·1030	0·1134	+9·17
,, kemp II	—	0·1013	0·1055	+3·98
,, (mixed sample of 6 g.)	3·25	0·5510 (Sullivan)	0·5908	+6·73
Welsh Mountain wool	4·08	0·1260	0·1255	−0·40
,, kemp	—	0·0975	0·0988	+1·32
,, birth-coat I	3·06	0·0957	0·0954	+0·31
,, birth-coat II	2·72	—	—	—
Scotch blackface kemp	—	0·0787	0·0811	+2·96

* $\frac{\text{Total S of hydrolysate as cystine} - \text{cystine (colorimetric)}}{\text{Total S as cystine}} \times 100.$

Discussion.

Only in the case of camel hair can it be said, on reference to Table I, that there is any significant difference between the cystine content of kempy material (medullated fibres) as determined directly and by calculation from the total sulphur content. The differences noted in the case of Welsh mountain wool, kemp and birth-coat are well within experimental error.

The series is somewhat limited on account of the difficulty of obtaining sufficiently large quantities of kemp from normally fine-woolled sheep. It appears however that kemp, containing a large proportion of medullary cells, resembles non-medullated sheep's wool in containing no significant quantities of any sulphur-containing amino-acid other than cystine.

Camel hair, both inner and outer coat, as previously found shows evidence of non-cystine, sulphur-containing compounds. In this connection it should

be remembered that these fibres contain a dark, reddish-brown pigment and it is not improbable that this pigment itself contains sulphur.

The total sulphur content of Welsh mountain lambs' birth-coat is very considerably less than that of the wool of the same breed, just as the "puppy coat" of rats is less rich in cystine and sulphur than the corresponding hair of the adult [Lightbody and Lewis, 1929]. Rabbits' fur also increases in sulphur content with increase in the age of the animal [Düring, 1897]. It appears, in agreement with Barritt and King [1926], that the medullary cells are less rich in cystine than are the cortical cells of the fibre.

Summary.

1. Samples of Scotch blackface kemp, Welsh mountain wool, kemp and lambs' birth-coat have been examined and in all cases practically the entire sulphur could be accounted for as cystine.

2. In the case of two separate samples of camel hair, both true hair (inner coat) and coarse outer-coat fibres contained more sulphur than could be accounted for as cystine. It is suggested that the brown-red pigment present in these samples may account, in part at any rate, for the discrepancy.

The author wishes to thank Mr A. T. King for his continued interest in the subject of this work, and Mr H. Rothera for valuable help in the preparation of the material.

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XI. COLOUR REACTIONS OF STEROLS WITH NITRIC ACID.

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(*Received December 9th, 1930.*)

In the course of experiments on the dehydrogenation of zymosterol by means of mercuric acetate, it was noticed that nitric acid, used for cleaning the reaction vessel, gave an intense blue colour reaction with fragments of the reaction mixture left in the flask. This chance observation led to a systematic study of the reaction of nitric acid with sterols. The characteristic colours produced with certain sterols were found to be somewhat transient when pure colourless nitric acid was employed. The oxidising action of the acid was retarded and the colour reactions were stabilised to a certain extent by using a mixture of glacial acetic acid with nitric acid (1:4). Finally it was found that nitric acid containing the acetates of metals (magnesium, cerium, cadmium, mercury, uranium, etc.) gave fairly stable colour reactions. They differed to some extent with different metals, and in order to simplify description the following notes are restricted to the reactions of the "mercury reagent." The reagent is prepared by dissolving 25 g. of mercuric acetate in 100 cc. of nitric acid (sp. gr. 1·42). Nitrous acid interferes with the colour reactions, and it is necessary to decolorise the solution with a few crystals of urea. When this precaution is used the reagent apparently keeps indefinitely.

The reaction is carried out by adding to a chloroform solution of the sterol an equal volume of the reagent, and shaking immediately. On account of the high specific gravity of the mercury reagent, the mixture separates rapidly into an upper coloured chloroform layer and a lower, usually colourless, layer of the reagent.

1. *Red colours* are given by *allo*-cholesterol, *allo*-sitosterol, cholesterilene, ψ-cholestene and β-cholesterol. The tint of the red colours varies somewhat with different sterols and is orange, carmine red or magenta according to concentration, but all the solutions show selective absorption in the region of $500\mu\mu$. A red colour is given only by those sterols which are believed to possess a $\Delta^{1,2}$ (or $\Delta^{1,13}$) ethenoid linkage in the molecule and which have been found previously [Rosenheim, 1929] to give a red colour reaction with trichloroacetic acid. It may be assumed therefore that the mechanism of the reaction consists, as in the latter case, in the formation of coloured carbonium salts and that a red colour reaction with the mercury reagent is specific for

and dependent on the presence of a $\Delta^{1,2}$ (or $\Delta^{1,13}$) linkage in the sterol ring system.

Considerations of the relationship of colour reactions and absorption spectra of sterols to their structure have led Heilbron and Spring [1930] to the conclusion that "selective absorption of sterols is conditioned by the presence of two ethenoid linkages in the molecule, one of which must apparently be in the $\Delta^{1,13}$ (or $\Delta^{1,2}$) position." The fact that oxycholesterilene possesses two ethenoid linkages and a highly selective absorption at $280\mu\mu$ [Rosenheim and Adam, 1929], but gives a negative reaction with both the mercury reagent and trichloroacetic acid (see later) weakens the evidence adduced by Heilbron and Spring for such a generalisation.

2. *A blue colour*, following a transient pink, is given by ergosterol and its esters when the reaction is carried out with dilute solutions (5 mg. or less in 1 cc. chloroform), a marked reaction still showing with 0·01 mg. The tint is a characteristic peacock-blue, and the solution does not show any selective absorption. On prolonged standing the blue colour changes through magenta into a dull shade of red. The lower acid layer rapidly loses its initial rose-red colour and becomes colourless.

An entirely different reaction results when relatively concentrated solutions of ergosterol (10 mg. or more in 1 cc. chloroform) are used. The same initial pink colour appears on mixing with the reagent, but it rapidly changes into orange and a clear yellow, instead of into blue. The acid layer remains colourless throughout.

The latter reaction has been found useful as an index of the purity of ergosterol, since oxidised or otherwise changed specimens give finally an intense green reaction under the above conditions [Callow, 1931]. The sensitiveness with which the reaction indicates such changes is demonstrated by allowing a 1 % chloroform solution of ergosterol to stand in a stoppered cylinder exposed to diffused daylight. Within a few hours the still colourless solution gives with the mercury reagent a purple and finally an intense green colour, showing a green fluorescence. The resemblance of this reaction to that of *iso*ergosterol (see later), and the observation that this change does not occur in solutions protected from light, suggests that it is due to the production of *iso*ergosterol. Under the influence of light, chloroform decomposes into phosgene and hydrochloric acid, and the latter is known to isomerise ergosterol into *iso*ergosterol at ordinary temperatures. The liberation of hydrochloric acid from chloroform has been shown to be the cause also of the change from red to green when a chloroform solution of bilirubin is exposed to sunlight [Thudichum, 1883].

Ergosterol peroxide [Windaus and Brunken, 1928] and dehydroergosterol peroxide [Windaus and Linsert, 1928] yield greenish-blue reactions similar to that of dilute ergosterol solutions.

The reaction of *iso*ergosterol [Reindel, Walter and Rauch, 1927] differs essentially from that of ergosterol. The colour changes end in olive green after

passing rapidly through red to purple and blue. In the blue stage a well defined absorption band at 550 $\mu\mu$ can be observed, and the final green stage shows a green fluorescence. A further distinction from ergosterol is seen in that the colour changes are identical in concentrated or dilute solutions. The influence of the complex Hg ion is evident in this reaction: when cadmium acetate replaces mercuric acetate, the *iso*ergosterol reaction stops at the red stage. The various *iso*ergosterols of Bills and Cox [1930] have not been investigated.

3. *Colourless solutions* are obtained in the reactions with (1) cholesterol and its derivatives (cholesteryl chloride, α-cholesterol oxide, α-cholestanetriol, cholestenone, oxycholestenone, oxycholesterilene, dihydrocholesterol), (2) coprosterol and coprostanone, (3) the phytosterols: stigmasterol, sitosterol, γ-sitosterol, dihydrositosterol, (4) zymosterol (impure preparations give an intense blue-green reaction, due to ergosterol), lanosterol (from lanolin) and β-amyrol, (5) dihydroergosterol and tetrahydroergosterol.

From these facts it is apparent that only those sterols can give colour reactions with nitric acid or with the mercury reagent which possess either the $\Delta^{1,2}$ (or $\Delta^{1,13}$) linkage or the unknown ethenoid linkages of the ergosterol molecule. The non-reactivity of the two partially reduced ergosterols is not surprising, since a shifting of the double linkages may have taken place during reduction. Both still contain an "inert" ethenoid linkage, which Heilbron and Spring [1930] connect with a positive bromine reaction (Tortelli-Jaffé). It may be suggested however that the latter reaction is a secondary one and connected with the destructive action of bromine on the ergosterol molecule. In favour of this view is the fact, found by us, that iodine replacing bromine in the Tortelli-Jaffé reaction, gives a green colour with ergosterol, but does not react with either dihydroergosterol or tetrahydroergosterol.

Other reactions. (1) *Vitamin A and carotene.* Most cod-liver oils give with the mercury reagent a series of indefinite colour reactions ending in red. Other liver oils, which are many times as rich in vitamin A as cod-liver oil [Rosenheim and Webster, 1927], such as certain mammalian liver oils, salmon-liver oil, etc., give a brilliant transient blue colour with the reagent. The unsaponifiable fraction of these oils, and also of cod-liver oil, yields an intense and rather more stable blue colour, which is apparently identical with the colour produced with the unsaponifiable fraction by $AsCl_3$, $SbCl_3$ and trichloroacetic acid [Rosenheim and Webster, 1926]. The relationship of these colour reactions to vitamin A remains still to be defined.

In view of the resemblance of the colour reactions of cod-liver oil to those of carotene, which originally suggested a relationship of the latter to vitamin A [Rosenheim and Drummond, 1925] it was not surprising to find that carotene also gives a blue colour reaction with the mercury reagent. In distinction from the trichloroacetic acid reaction of carotene, the reaction is a transient one.

(2) *Vitamin D.* Irradiated ergosterol, freed from unchanged ergosterol

by digitonin, gives a transient red colour, followed by a permanent olive green with both the mercury reagent and trichloroacetic acid. Since the same reaction is given by biologically inactive, over-irradiated or oxidised specimens, it is probably due to one or more of the inactive substances produced from ergosterol by irradiation.

(3) *Bile acids and their derivatives.* Desoxycholic acid and cholic acid possess the same ring system as the saturated sterols and were accordingly found to be non-reactive. On distillation they form, by loss of water, the unsaturated choladienic and cholatrienic acids [Wieland and Weil, 1912], specimens of which we owe to the kindness of Professor Wieland. Whilst choladienic acid is non-reactive, cholatrienic acid in dilute chloroform solution gives with the mercury reagent a gentian-blue colour reaction, the lower acid layer turning deep red. An intense blue colour slowly develops with trichloroacetic acid on standing for a few hours, the blue solutions in both reactions showing a band in the red region of the spectrum with a maximum at 650 $\mu\mu$. It seems therefore that the ethenoid linkages connected with C_3 and C_7, which are common to both acids, are unable to react, and that the colour reaction of cholatrienic acid depends on the presence of the $\Delta^{11,12}$ or $\Delta^{12,13}$ linkage.

(4) "*Oxycholesterol*," the mixture of substances obtained by debromination of cholesterol dibromide with sodium acetate [Lifschütz, 1919] or by treatment of cholesterol with benzoyl peroxide, gives with the mercury reagent an immediate gentian-blue colour, showing the characteristic "oxycholesterol" absorption band at 650–680 $\mu\mu$. Traces of this product, sufficient to be detected by the reagent, are formed from cholesterol under all conditions where oxidation may take place. A positive reaction is given by cholesterol after recrystallisation in the presence of charcoal, after irradiation in air or after heating to the melting point in air, etc. Since "oxycholesterol" gives an identical reaction also with trichloroacetic acid, $AsCl_3$ or $SbCl_3$ [Rosenheim, 1927, 1929], and thus behaves like an unsaturated sterol, it may be suggested that the substance contains an additional ethenoid linkage, and is formed by dehydrogenation of cholesterol. Such a suggestion is strengthened by the ease with which oxidising agents like perbenzoic acid dehydrogenate sterols [Windaus and Lüttringhaus, 1930].

Summary.

1. Nitric acid gives characteristic colour reactions with certain sterols. A solution of mercuric acetate in nitric acid ("mercury reagent") is recommended as a general reagent.

2. A red colour with the mercury reagent indicates the presence of the $\Delta^{1,2}$ (or $\Delta^{1,13}$) linkage in sterols.

3. A greenish-blue colour is given by ergosterol in dilute solutions, whilst a yellow colour results when concentrated solutions of ergosterol are employed. The latter reaction serves as a useful index for the purity of ergosterol.

4. A gentian-blue colour is given by products containing vitamin A (certain liver oils and the "unsaponifiable" of cod-liver oil).

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XII. THE PURIFICATION OF ERGOSTEROL.

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Investigations proceeding in this laboratory on the formation of vitamin D made it necessary to obtain ergosterol in as pure a condition as possible. Great variations are recorded in the literature in the physical constants of ergosterol which has been the starting material for chemical and biochemical investigations, and it was considered essential to check the criteria of purity put forward by Tanret [1908] and subsequent workers. Although ergosterol of a high degree of purity may be obtained from certain specimens of crude yeast ergosterol by recrystallisation from a mixture of alcohol and benzene (2:1), as described by Bills and Honeywell [1928], the use of this solvent does not invariably yield products with equally high specific rotation [*cf.* Bills and Cox, 1929; Heilbron, Sexton and Spring, 1929]. According to the experience gained in this laboratory, recrystallisation from alcohol-benzene is extremely useful for the removal of zymosterol from ergosterol, but fails to free it from α-dihydroergosterol. α-Dihydroergosterol appears to be a normal constituent of the sterol mixture occurring in yeast [Callow, 1930, 1931], and its presence in varying proportions accounts almost entirely for the differences in the physical constants of ergosterol recorded in the literature. It is however removed by the method of fractional recrystallisation of the benzoates described here, and ergosterol from different sources, both British and Continental, has yielded purified specimens with no significant differences in their physical properties. No support is therefore given to the conclusion of Bills and Cox [1929] and Bills and McDonald [1930] that natural isomerides of ergosterol separable only with difficulty commonly occur in specimens of different specific rotation obtained from yeast grown under different conditions.

In the course of the preliminary investigations, ergosterol was distilled under reduced pressure. The conclusion of Reindel and Detzel [1929] that this is useless as a method of purification was confirmed.

Purification by means of the recrystallisation of an ester was then considered. The acetate [Windaus and Grosskopf, 1922] and the *iso*butyrate [Bills and Honeywell, 1928] have previously been used for this purpose. Preliminary experiments with ergosteryl ethyl carbonate, prepared from ergosterol by the action of excess of ethyl chloroformate in pyridine solution, indicated that this ester was not a suitable one. The benzoate, however, which has the advantage of being prepared under mild conditions of reaction, gave

an immediate separation into fractions of different specific rotation. Benzoylation of different specimens of yeast ergosterol was carried out with benzoyl chloride in pyridine under uniform conditions, as described in detail below, and the benzoates, which underwent an initial fractionation in the process of separation, were then further fractionally recrystallised from ethyl acetate. Purification was rapid when fractions were collected at 37°, and the head fractions attained a constant specific rotation after five or six recrystallisations at this temperature. The sterol was obtained from the purified benzoate by hydrolysis with alcoholic potassium hydroxide, and freed from traces of decomposition products by recrystallisation.

Three crystalline forms of ergosteryl benzoate were observed. It usually crystallises as a bulky mass of fine needles, a form which is actually stable only at high temperatures. The modification stable at ordinary temperatures forms rectangular plates. Rapidly cooled solutions deposit leaflets belonging to the monoclinic system, which soon pass into a mixture of the other two forms.

The specific rotations of the specimens of purified ergosteryl benzoate and ergosterol were concordant within the limits of the probable errors of determination. In Table I are given the values obtained, together with the melting-points and the figures recorded in the literature.

Table I.

Ergosterol [α] in chloroform			Ergosteryl benzoate		
M.P.	Anhydrous	Hydrated	M.P.	[α] in chloroform	Observer
165°	$[\alpha]_D$ −132°	$[\alpha]_D$ −126°	—	—	Tanret [1908]
166–183°	$[\alpha]^{20}_{5461}$ −170·6°	—	164–168°	$[\alpha]^{25}_D$ −177°*	Bills and Honeywell [1928]
	$[\alpha]^{20}_D$ −132°				
	—	—	168–170°	$[\alpha]^{23}_D$ − 68°	Windaus and Rygh [1928]
160–161°	$[\alpha]^{20}_D$ −133·1°	—	168–170°	$[\alpha]^{23}_D$ − 71°	Wieland and Asano [1929]
160–163°	$[\alpha]^{20}_{5461}$ −174·2°	$[\alpha]^{20}_{5461}$ −167·2°	169–171·5°	$[\alpha]^{20}_{5461}$ − 88·3°	Callow
	$[\alpha]^{20}_D$ −135°	$[\alpha]^{20}_D$ −128·7°	—	—	—

* This exceptional value is not a misprint, as suggested by Windaus and Rygh [1928]; *cf.* Bills and McDonald [1930].

The values of the specific rotation now recorded are slightly higher than those found originally by Tanret [1908] for ergosterol from ergot. The molecule of water of crystallisation in ergosterol crystallised from aqueous alcohol is difficult to remove completely without decomposition. The specific rotation of the hydrated sterol which has been kept over calcium chloride for 3–4 days is the most reliable index of its purity, accompanied by a confirmatory determination of the loss of weight on heating. Prolonged storage of ergosterol over a drying agent causes dehydration which is followed by rapid oxidation in air. In sealed tubes it is unchanged for long periods.

Experimental.

Material.

The following data were obtained for the samples of yeast ergosterol used for this work: (A) m.p. 160–162°, $[\alpha]^{20}_{5461}$ − 156·9°; (B) m.p. 159–162°, $[\alpha]^{20}_{5461}$ − 157·7°; (C) m.p. 160–163°, $[\alpha]^{20}_{5461}$ − 156·6°; (D) m.p. 160–163°, $[\alpha]^{20}_{5461}$ − 158·4°; (E) m.p. 160–163°, $[\alpha]^{20}_{5461}$ − 161·2°; (F) m.p. 155–160·5°, $[\alpha]^{20}_{5461}$ − 137·2°. The specimens were from four different sources, and all except the last had been recrystallised from alcohol-benzene at least once. The melting-points were determined in sealed capillary tubes. All specific rotations recorded here were measured in chloroform containing alcohol (B.P.) in a concentration of about 1 % in a 4 dm. tube, the solutions being generally made up at 20° and measured at the same temperature with an instrument reading to 0·01°. Even purified ergosterol melts over a range of temperature in a capillary tube, and the melting-point is a less reliable and sensitive index of purity than the specific rotation.

One specimen of ergosterol (A), distilled from a flask in a metal-bath at 260–270°/0·1–1·0 mm., gave a pale yellow distillate, $[\alpha]^{20}_{5461}$ − 145·3°. A purified sample of ergosterol, $[\alpha]^{20}_{5461}$ − 164°, began to distil at 192°/0·001–0·01 mm., and the bulk passed over at 198° (bath at 250–260°). A sublimate began to come off at an appreciable rate at a bath temperature of 180°. The distillate was white, and had m.p. 158–161·5°, $[\alpha]^{20}_{5461}$ − 164·1°; the specific rotation had therefore fallen, since the distillate was anhydrous. Recrystallised from alcohol-benzene (2:1) it had $[\alpha]^{20}_{5461}$ − 161·2°.

Another specimen of ergosterol (F) was fractionally sublimed under the conditions used for distillation of vitamin D [Askew *et al.*, 1930]. Fractions were obtained from 0·5 g. at 135° (0·062 g., m.p. 160–164°, $[\alpha]^{20}_{5461}$ − 135°), at 145° (0·044 g., m.p. 156–163°), at 155° (0·317 g., m.p. 156·5–163°, $[\alpha]^{20}_{5461}$ − 148°), and a residue was left (0·06 g., m.p. 154·5–161·5°). These results did not justify further work.

The distilled materials were hygroscopic and very readily oxidised in air. Over calcium chloride in a desiccator, kept in a cupboard away from bright light, and opened at intervals, weighed samples turned yellow and gained in weight owing to absorption of oxygen. Reindel and Detzel [1929] made a similar observation, but, contrary to their statement, no break was observed in the curve of increase in weight at a composition corresponding to a "labile moloxide," $C_{27}H_{42}O_3$. The absorption of oxygen, after a perceptible initial acceleration, went on at a continuously decreasing rate until practical constancy of weight was attained after a year at an increase of 19·5–21 %, corresponding to the addition of five atoms of oxygen (calculated, 20·9 %). The measurements are illustrated by the curve in Fig. 1. It seems probable that this rapid oxidation is due to the combined effects of autocatalysis, fine state of division, and complete initial dehydration. Samples of recrystallised ergosterol kept under the same conditions show a slight initial loss of weight followed, after two or three months, by an increase in weight, the rate of

which at first increases and then decreases, accompanied by a deepening yellow colour. One sample, after a year, gained 18 % in weight, and was still gaining. It appeared likely to reach the same limit as the distilled products

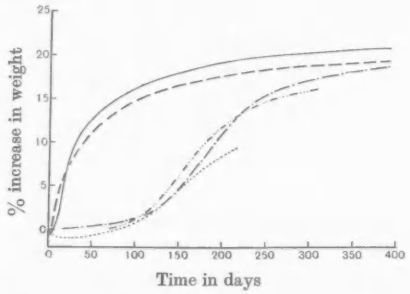

Fig. 1. Increase in weight of ergosterol kept over $CaCl_2$ in air.

——— Distilled, purified ergosterol. — — — Distilled ergosterol (A).
····· Ergosterol (E).
·—·—· Ergosterol (A), recrystallised from alcohol-benzene.
- - - - - - - Ergosterol purified *via* the benzoate.

after a further six months. The nature of the products formed has not yet been investigated.

Ergosteryl ethyl carbonate. Ergosterol (3·4 g.) was dissolved in dry pyridine (60 cc.) and ethyl chloroformate (14 cc.) was added slowly with constant shaking and cooling. After standing for 30 minutes the mixture was poured into water and the precipitated solid was collected and washed. Much frothing occurred in both stages of the preparation. Recrystallised from absolute alcohol and from ethyl acetate, *ergosteryl ethyl carbonate* was obtained as thin plates, M.P. 150–153·5°, $[\alpha]^{20}_{5461} - 111{\cdot}1°$. The yield was 2 g. (Found (micro): C, 79·3; H, 10·4 %. $C_{30}H_{46}O_3$ requires C, 79·3; H, 10·1 %.) The ultra-violet absorption spectrum in alcoholic solution closely resembled that of ergosterol. When the carbonate was irradiated in solution under the same conditions as ergosterol, the product showed equal antirachitic activity. The ester, in this respect, thus resembles the acetate.

A small fraction of the crude product was insoluble in alcohol, and crystallised from ethyl acetate in needles, M.P. 206–212°. Fractionation of the alcoholic mother-liquors showed the presence of unchanged ergosterol. Hydrolysis of the ester with alcoholic potassium hydroxide yielded ergosterol, which, recrystallised from 95 % alcohol, had M.P. 159–162°, $[\alpha]^{20}_{5461} - 164{\cdot}2°$. No further work was done with the ergosteryl ethyl carbonate, since the conditions of its preparation were less satisfactory than those of the benzoate, and it had no advantage with respect to purification.

Preparation of ergosteryl benzoate. Ergosterol (1 part) in dry pyridine (20 volumes) was treated with redistilled benzoyl chloride (2·5–3 volumes) in small portions while the flask was shaken and cooled under the tap. The

development of a permanent pink colour was a reliable indication of the presence of a slight excess of benzoyl chloride. It was necessary to use much more than the calculated quantity of benzoyl chloride in order to obtain complete esterification, and the excess required varied considerably with different specimens, probably according to the amount of solvent of crystallisation or the degree of dryness of the pyridine. Under these conditions part of the benzoate crystallised out directly from the reaction mixture, together with pyridine hydrochloride. The necessity of pouring the whole into water was obviated, and a fractionation was obtained. After cooling to room temperature, the solid which separated (fraction I) was collected and washed with a very little pyridine, and the filtrate was cooled to $-4°$. Fraction I was, in the meantime, washed thoroughly with water and finally with alcohol. Fraction II, obtained at $-4°$, was treated similarly, and the aqueous washings from the two crops (in all 40 volumes) were added to the filtrate. The precipitate thus produced (fraction III) was collected and washed. Ethyl benzoate, in some cases in large amount, separated with the precipitate. In the light of subsequent work, it is evident that this was derived from the alcohol of crystallisation of the α-dihydroergosterol originally present. Pyridine was recovered from the filtrate.

In a typical preparation sample D of ergosterol (45 g.) gave the following fractions: (I) 35·8 g., M.P. 164–171°, $[\alpha]^{20}_{5461} - 86{\cdot}8°$; (II) 7·1 g., M.P. 163–168·5°, $[\alpha]^{20}_{5461} - 82{\cdot}9°$; (III) 7·0 g., M.P. 135–150°, $[\alpha]^{20}_{5461} - 36°$; total yield, 91 %.

Purification of ergosteryl benzoate. In general, fractions I and II were worked up in the same scheme of fractional crystallisation, whilst fraction III was treated separately for the isolation of admixed sterols whose presence was suspected. The system ultimately adopted was to recrystallise fraction I repeatedly until no further alteration in specific rotation took place, and to utilise the mother-liquors for the recrystallisation of subsequent batches of crude benzoate. In this way there accumulated mother-liquors in which impurities of the original ergosterol collected. The ethyl acetate used as solvent was freed from acetic acid and higher esters and dried. The crude benzoate was dissolved, in a current of nitrogen, in 25–35 volumes of boiling ethyl acetate to give a nearly saturated solution, which was then allowed to cool in a closed flask to 37° in the hot room. It was found in the first stages of the work that crystallisation at 37° rather than at room temperature gave a much more efficient purification, and much reduced the number of recrystallisations necessary. Oxidation took place to an undesirable extent in the mother-liquors if hot solutions were exposed freely to the air. A mixture of butyl alcohol and benzene (2:1), and 80 % aqueous pyridine were also tried as solvents, but had the disadvantage of being difficult to remove and of hindering the recovery of material from mother-liquors. Pyridine seemed particularly to favour decomposition and oxidation. Ethylene dichloride was unsuitable because of the difficulty of keeping it free from traces of hydrogen chloride,

to which ergosterol is very sensitive. None of these solvents, moreover, gave evidence of further purification after exhaustive crystallisation from ethyl acetate.

The course of the purification of the benzoate from sample F may be taken as an example. Fraction I, twice recrystallised from ethyl acetate at 37° yielded material (1), $[\alpha]^{20}_{5461} - 83{\cdot}7°$. Crystallisation of this (33 g.) from ethyl acetate (1100 cc.) yielded (2), $[\alpha]^{20}_{5461} - 86{\cdot}2°$ (20 g.), and so, in succession, (3), $[\alpha]^{20}_{5461} - 86{\cdot}7°$ (10·8 g.); (4), $[\alpha]^{20}_{5461} - 87{\cdot}2°$ (6·4 g.); (5), $[\alpha]^{20}_{5461} - 88{\cdot}1°$ (4 g.); (6), $[\alpha]^{20}_{5461} - 88{\cdot}0°$ (2·8 g.). In another case (sample D above), the successive fractions were: (1) M.P. 167–172°, $[\alpha]^{20}_{5461} - 87{\cdot}6°$; (2) (from butyl alcohol-benzene) M.P. 167–171·5°, $[\alpha]^{20}_{5461} - 88{\cdot}0°$; (3) M.P. 166–171·5°, $[\alpha]^{20}_{5461} - 88{\cdot}5°$; (4) (from butyl alcohol-benzene) M.P. 169–171·5°, $[\alpha]^{20}_{5461} - 88{\cdot}7°$. A third specimen of benzoate (from samples B and C), after purification, had $[\alpha]^{20}_{5461} - 88{\cdot}0°$, and then, in two successive recrystallisations from ethyl acetate, gave material having $[\alpha]^{20}_{5461} - 88{\cdot}15°$, and $[\alpha]^{20}_{5461} - 88{\cdot}2°$. Comparable figures were obtained in all cases for the benzoate recovered from the mother-liquors of the head fractions, separated in later stages of the fractionation. Thus, after five or six recrystallisations from ethyl acetate, a pure, homogeneous benzoate, having a mean specific rotation $[\alpha]^{20}_{5461} - 88{\cdot}3°$, without significant variation, was obtained from all the samples of ergosterol.

Rectangular plates accompanied the usual needles of ergosteryl benzoate, as recorded by Wieland and Asano [1929], most frequently when the mother-liquors of fractions collected at 37° were allowed to stand at room temperature. It was ultimately discovered that from butyl alcohol-benzene solutions, the separation of either plates or needles could be obtained by seeding with the required form, although rapid cooling favoured the separation of plates, and slow cooling the separation of needles. Both forms were obtained from the purified benzoate with the same specific rotation, $[\alpha]^{20}_{5461} - 88{\cdot}4°$. No attempt was made to determine the transition point of the two forms, but the slow replacement of needles by plates in the presence of various solvents shows that the plate form is the stable one at room temperatures. A specimen of plates began to melt at 165° to give a cloudy mixture, in which a mass of fine needles could be discerned with the aid of a lens, and became clear at 170°, so that the needle form is evidently the stable one at high temperatures. A third crystalline form was observed when solutions of the benzoate were cooled rapidly without agitation. Leaflets separated which were replaced by a felted mass of needles and a few plates when the mixture was stirred. Isolation of the leaflets in bulk was impossible, but in portions of the solution in butyl alcohol-benzene transferred to a microscope slide it was possible to observe hexagonal leaflets, belonging to the monoclinic system, which dissolved and were replaced by rectangular plates and a few needles, the latter afterwards gradually dissolving.

Ultra-violet irradiation of ergosteryl benzoate gave a product with about 1/8 the antirachitic activity of ergosterol similarly treated, but the activity

rose to that of the irradiated free sterol when the product was hydrolysed [*cf.* Windaus and Rygh, 1928].

Hydrolysis of ergosteryl benzoate. This was done by heating the benzoate with 25 parts of 3 % alcoholic potassium hydroxide and boiling for 5 minutes after solution was complete. As a precaution against oxidation, the reaction was carried out in an atmosphere of nitrogen. The ergosterol which separated on cooling was collected, washed with alcohol and water, recrystallised from 40–50 parts of 95 % alcohol, and dried for several days over calcium chloride. The yield was about 67 %, and a further 15 %, generally of slightly lower rotation, could be obtained by working up the mother-liquors.

Properties of purified ergosterol. The characteristics of the pure ergosterol thus obtained from different samples were as follows:

(A) M.P. 160–162°, $[\alpha]^{15}_{5461}$ – 165°, loss at 100°/12 mm., 4·5 % (calculated for $C_{27}H_{42}O$, H_2O: 4·5 %);

(B + C) M.P. 160–163°, $[\alpha]^{20}_{5461}$ – 166·8°, – 167·7°, – 167·2° (different batches; mean, – 167·2°), $[\alpha]^{20}_{D}$ – 128·7°, anhydrous substance, $[\alpha]^{20}_{5461}$ – 174·2°, $[\alpha]^{20}_{D}$ – 135°, loss at 120°/0·1 mm., 4·5 %;

(D) M.P. 160–163°, $[\alpha]^{20}_{5461}$ – 166·5°, loss at 120°/10 mm., 4·7 %;

(F) M.P. 160–163°, $[\alpha]^{20}_{5461}$ – 165·9°.

Dehydration of ergosterol by heating under reduced pressure in an Abderhalden "pistol" with steam or xylene vapour was accompanied by slight decomposition, and the specific rotation was a little below the calculated value. The material was generally slightly sintered and had acquired a yellow tinge. Less decomposition occurred when the ergosterol was quickly heated to just above its melting-point in a current of pure, dry nitrogen. The anhydrous material gained in weight rapidly, owing to oxidation, when exposed to the air. Purified ergosterol gained weight in a similar way to unpurified ergosterol when kept over calcium chloride (see Fig. 1). No change in specific rotation or melting-point was found after six months in a specimen kept in the dark in a sealed, evacuated tube.

Summary.

1. Distillation of ergosterol and recrystallisation of ergosteryl ethyl carbonate are unsatisfactory methods for purifying ergosterol, and recrystallisation from alcohol-benzene (2:1) does not invariably yield a pure product.

2. A high degree of purity is attained by benzoylation of ergosterol, recrystallisation of the benzoate from ethyl acetate at 37°, and hydrolysis.

3. Ergosteryl benzoate is trimorphic.

4. No variation has been found in the properties of purified yeast ergosterol from different sources, and no evidence of the existence of natural isomerides has been obtained.

5. When ergosterol is kept over a dehydrating agent in air it takes up five

atoms of oxygen. This oxidation is rapid in the case of distilled material, but also takes place with recrystallised hydrated material after an induction period.

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XIII. OCCURRENCE OF α-DIHYDROERGOSTEROL AS AN IMPURITY IN YEAST ERGOSTEROL.

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The fractionation of the benzoates obtained from ordinary ergosterol [Callow, 1930, 1931] has led to the isolation of α-dihydroergosterol, the occurrence of which among the yeast sterols has not hitherto been reported. This compound is the only one found present in sufficient amount for separation and identification.

The attempt to isolate α-dihydroergosteryl benzoate by means of fractional crystallisation was a laborious process. The more soluble fractions of the benzoates from ordinary ergosterol crystallised from ethyl acetate in stable leaflets isomorphous with the unstable form of ergosteryl benzoate, and the properties of the material closely resembled those of neosteryl benzoate [Wieland and Asano, 1929]. On repeated recrystallisation from other solvents, however, the laevorotation diminished and the melting point rose, whilst the product still contained a large proportion of ergosteryl benzoate. It became evident that the unstable leaflet form of ergosteryl benzoate formed a stable mixed crystal phase with another benzoate crystallising in isomorphous leaflets. After the accumulation of larger quantities of material an attempt was made to remove the ergosterol by fractional crystallisation from ethylene dichloride, which was the best of the various solvents tried, but the results indicated that the isolation of any considerable quantity by this method would be impracticably slow. Recourse was had therefore to a chemical method of separation. It was found that partial bromination decomposed the ergosteryl benzoate preferentially, and ultimately the ergosterol content of the product of hydrolysis was reduced to 0·25 %. The last traces of ergosterol could be removed by ultra-violet irradiation.

The identity of the sterol thus isolated with α-dihydroergosterol, indicated by the correspondence of the properties with those recorded in the literature, was confirmed by comparison with an authentic specimen. The *p*-nitrobenzoate, which was prepared for the first time, is remarkable for the large range of temperature (46°) over which the anisotropic liquid phase is stable. This range is small in all derivatives of ergosterol hitherto examined, and the crystalline liquid is difficult to observe at all in some cases [*cf.* Gaubert, 1908].

No indication of the presence of sterols other than α-dihydroergosterol was obtained, although their presence in small amount in the most soluble

fractions is not impossible. The similarity of the neosteryl benzoate of Wieland and Asano [1929] to the products obtained in the initial stages of the separation of α-dihydroergosteryl benzoate strongly suggests that the former material was a similar isomorphous mixture. The data given, in particular the absorption spectrum, and the comments made by Wieland and Gough [1930] give some support to this suggestion. It was actually found that a mixture of ergosterol and α-dihydroergosterol yielded a benzoate, resembling neosteryl benzoate, which formed homogeneous crystals from ethyl acetate and showed little change in specific rotation after recrystallisation from that solvent. The existence of neosterol as a chemical entity cannot of course be denied without examining a sterol mixture similar to that used by Wieland and Asano as their starting product. The "cerevisterol" of Bills and Honeywell [1928], which would be expected to yield a benzoate of high melting point and slight solubility, was not present, nor has any indication of its presence been obtained in numerous other specimens of ergosterol in this laboratory.

It is evident that in the specimens of ergosterol examined the variations in specific rotation could be accounted for almost entirely by the presence of α-dihydroergosterol in varying amounts, and that this is the only considerable impurity. The specific rotation of a mixture of 95 % of ergosterol (+ H_2O; $[\alpha]^{20}_{5461}$ − 167·2°) with 5 % of α-dihydroergosterol (+ 0·5 C_2H_5OH; $[\alpha]^{20}_{5461}$ − 21·1°) would be $[\alpha]^{20}_{5461}$ − 159·9°, which is close to the average values shown by ordinary "pure" ergosterol.

α-Dihydroergosterol shows absorption only in the extreme ultra-violet, and it is unaffected by radiation of wave-lengths above 275 μμ. Windaus, Gaede, Köser and Stein [1930], in a recent paper describing the products of over-irradiation of ergosterol through "Uviolglas," record that 4 % of the product was precipitated as a digitonide which proved to be that of α-dihydroergosterol. In the light of the results now described it is probable that the latter was present in their starting material.

The recognition of α-dihydroergosterol as a companion of ergosterol in yeast indicates that the simultaneous occurrence of sterols in different stages of saturation is a general phenomenon connected with the synthetic cellular activity of plants and animals. Evidence for such a view has been accumulating during the course of this work. The occurrence of dihydroergosterol along with fungisterol and ergosterol in ergot [Heyl and Swoap, 1930] is analogous to its association with zymosterol and ergosterol in yeast. Dihydrositosterol has been found to be a constant companion of sitosterol in cereals [Anderson, Nabenhauer, and Shriner, 1927] and in the soya bean [Bonstedt, 1928]. The latter also contains a doubly unsaturated sterol, stigmasterol, which is found associated with sitosterol in the Calabar bean and cacao. Finally, it has recently been found [Schönheimer, Behring, Hummel, and Schindel, 1930], that the main sterol constituent of animal cells, cholesterol, contains dihydrocholesterol as a constant admixture. The significance of the occurrence of these saturated sterols is uncertain. The last named authors consider that dihydro-

cholesterol is formed by hydrogenation of cholesterol, but there is as yet little evidence to indicate whether the formation of the more saturated sterols by reduction is general. It is possible that in some cases they are intermediates in the formation of the unsaturated compounds.

Experimental.

Material. The specimens of ergosterol which were the starting material for this work were the same as those used in the purification of ergosterol previously described [Callow, 1931]. After benzoylation of the crude ergosterol with benzoyl chloride in pyridine and separation of the material which crystallised out directly, a final fraction (10–15 % of the total yield) was obtained by the addition of water to the pyridine solution. The presence of the benzoate of another sterol was immediately evident when this fraction was recrystallised from ethyl acetate, for a new stable crystalline form appeared—homogeneous leaflets belonging to the monoclinic system in the case of the least pure specimen of ergosterol (F; $[\alpha]^{20}_{5461} - 137{\cdot}2°$), and mixtures of leaflets with needles of ergosteryl benzoate in the case of other specimens ($[\alpha]^{20}_{5461} - 156{\cdot}9$ to $- 161{\cdot}2°$). In addition, the higher fractions of the benzoates from specimen F yielded mixtures of needles and leaflets, and a small quantity of leaflets was obtained from the mother-liquors of the higher fractions of the benzoates from other specimens. Mixtures of leaflets and needles could be separated by dissolving in boiling ethyl acetate and collecting fractions at 37° and − 4°. In this way batches of leaflets were collected with M.P. ranging from 159–165° to 165–170°, and values of $[\alpha]^{20}_{5461}$ ranging from − 59° to − 65°. In a typical case 50 g. of specimen A of ergosterol gave a total of 6·8 g. of leaflets.

Some difficulty was encountered in the initial stages of the work owing to the change which took place in ethyl acetate mother-liquors kept exposed to the air. This change, the nature of which is still uncertain, was indicated by an increase in solubility, yellowing of the solution on concentration, and the subsequent deposition of poorly crystallised products. The lower crystalline fractions from ethyl acetate also showed signs of oxidation when stored in contact with air. Simultaneously, the material developed an intense colour reaction with a 25 % solution of mercuric acetate in nitric acid ("mercury reagent") [Rosenheim and Callow, 1931]. When a chloroform solution of the material was mixed with the reagent and shaken the chloroform layer turned bluish-red, changing to blue, and then, after an interval, to green, the acid layer remaining colourless. In the ultimate systematic fractionation the composition of all fractions obtained was checked by the use of this reagent.

After hydrolysis with 3 % alcoholic potassium hydroxide these fractions of altered material yielded a brown solution from which a colourless sterol mixture separated, and the latter, when re-benzoylated, gave a mixture of benzoates which showed only the usual ergosterol reaction with the "mercury reagent" (chloroform layer pink → orange → yellow). The difficulty of working up sterol mixtures from the mother-liquors of technical ergosterol may be due

to the presence of similar transformation products of ergosterol, and the re-saponification process which Wieland and Gough [1930] applied to such material may owe its usefulness to the destruction of these products by alkali.

Separation of mixtures rich in α-dihydroergosteryl benzoate by fractional crystallisation. After preliminary investigations which indicated that the fractions of the benzoate crystallising in leaflets contained a large proportion of ergosteryl benzoate, and after trials with various solvents, a systematic fractionation was begun with 180 g. of material by crystallisation from ethylene dichloride. The benzoate was dissolved in eight parts of boiling ethylene dichloride in an atmosphere of nitrogen, cooled, and fractions were collected at 37° and at − 4°. The mother-liquors were either used for the recrystallisation of lower fractions or were evaporated in a current of nitrogen. In this way at one end of the system a series of less soluble fractions was obtained with decreasing laevorotation and rising M.P., whilst ergosteryl benzoate became concentrated in the more soluble fractions and ultimately separated in needles. The progress of the separation is illustrated by the following figures for successive head fractions: (1) M.P. 176–181°, $[\alpha]^{20}_{5461}$ − 55·8°; (2) M.P. 179–183·5°, $[\alpha]^{20}_{5461}$ − 45·5°; (3) M.P. 180·5–187°, $[\alpha]^{20}_{5461}$ − 38·7°; (4) M.P. 185–190°, $[\alpha]^{20}_{5461}$ − 32·2°, 12·1 g.; (5) M.P. 187·5–192°, $[\alpha]^{20}_{5461}$ − 26·6°, 6·3 g. When the M.P. and specific rotation were plotted on curves against number of recrystallisations it appeared that at least six further recrystallisations would be necessary before practical constancy could be attained.

Removal of ergosterol by bromination. Bromination of the mixed benzoates was initially tried in the hope that the sterol, then unidentified, accompanying ergosterol would form an easily separable bromide. Preliminary experiments, however, in which bromination was carried out in ether, chloroform or carbon tetrachloride, yielded no product sparingly soluble in alcohol when excess of bromine was used. When smaller proportions of bromine, preferably in carbon tetrachloride solution, were used, the addition of alcohol precipitated products free from bromine, and these were found to have the same properties as the products obtainable from the same starting material by fractional crystallisation.

The following method was found suitable for removing ergosterol. The mixture of benzoates (30·7 g.; containing about 30 % of ergosterol) was dissolved in carbon tetrachloride (400 cc.), and a solution of bromine in carbon tetrachloride (31 cc. of a 5 % solution by volume; about 2·5 mols./mol. of ergosterol) was added slowly with shaking. After allowing to stand for a few minutes, 95 % alcohol (1500 cc.) was added. The precipitate (18·8 g.) was collected, washed with alcohol, and recrystallised from ethylene dichloride. The slightly discoloured product had M.P. 189–193°, $[\alpha]^{20}_{5461}$ − 12°, and contained 3 % of ergosterol, determined colorimetrically. The ergosterol content was not reduced below 1 % by repeating the bromination. A specimen obtained in this way contained 1 % of ergosterol, and had M.P. 194–196°, $[\alpha]^{20}_{5461}$ − 13·0°. (Found (micro): C, 83·4; H, 10·0 %; $C_{34}H_{48}O_2$ requires C, 83·5;

H, 9·9 %.) It was found best, when dealing with fractions of high ergosterol content, to remove the ergosterol by two successive partial brominations.

α-Dihydroergosterol. Hydrolysis of the benzoate (14 g.; 3 % ergosteryl benzoate) was carried out by boiling for 45 minutes with 3 % alcoholic potassium hydroxide solution (800 cc.). Water was added to the hot solution until it was slightly cloudy, and the crystals which separated on cooling were collected, washed, dried, and recrystallised from alcohol-benzene (2:1). α-Dihydroergosterol (8·6 g.), containing 0·25 % of ergosterol, separated in leaflets, M.P. 172–174°, containing 0·5 mol. of alcohol of crystallisation. The solvent-free substance had $[\alpha]^{20}_{5461} - 23{\cdot}8°$. (Found: loss at 130°/10 mm. over P_2O_5, 5·7, 5·7 %, and, in solvent-free substance (micro), C, 83·6; H, 11·6 %. $C_{27}H_{44}O$, 0·5 C_2H_5OH requires loss, 5·65 %. $C_{27}H_{44}O$ requires C, 84·3; H, 11·5%.) The digitonide was precipitated by the addition of 1 vol. of a 1 % solution of digitonin in 90 % alcohol to 2 vols. of a 0·1 % solution of the sterol in 90 % alcohol. (Found: digitonide: sterol = 3·9:1.)

A portion of this specimen of α-dihydroergosterol was irradiated in 0·5 % solution in benzene by a quartz mercury vapour lamp for a period sufficient to destroy the ergosterol present. After evaporation and crystallisation of the residue twice from 95 % alcohol (charcoal), the product, freed from solvent at 130°/10 mm. (loss 5·9 %), had M.P. 172·5–174° (unchanged by mixture with an authentic specimen of α-dihydroergosterol of the same M.P.), and $[\alpha]^{20}_{5461} - 23{\cdot}0°$. (Windaus and Brunken [1928] give M.P. 173–174°, $[\alpha]^{17}_{D} - 20{\cdot}4°$; Heilbron and Sexton [1929] give M.P. 173°, $[\alpha]^{20}_{5461} - 21{\cdot}7°$.) The colour reactions were as follows: Liebermann-Burchard: momentary purple → dark-blue → bottle-green → emerald. Bromine (Tortelli-Jaffé): negative in dilute solution; in concentrated solution, yellow → emerald → blue-green. Iodine [Rosenheim and Callow, 1931]: negative. Salkowski: chloroform colourless, acid deep yellow with green fluorescence. Antimony trichloride: negative, red after standing. Trichloroacetic acid: negative, dull green with light green fluorescence after 6 hours. Mercuric acetate in nitric acid [Rosenheim and Callow, 1931]: negative. The slow, secondary reactions with antimony trichloride and trichloroacetic acid, which have not been described before, were also given by an authentic specimen of α-dihydroergosterol. The digitonide was precipitated in 90 % alcohol. (Found: digitonide: sterol = 4:1.)

Spectrographic examination of purified α-dihydroergosterol in 0·1 % alcoholic solution showed that absorption was negligibly small at wave-lengths exceeding 250 μμ, but then increased rapidly down to 230 μμ, the limit of measurement [*cf.* De Boe, 1930]. Investigation of the effect of irradiation in alcoholic solution by a quartz mercury vapour lamp with and without filters [*cf.* Askew *et al.*, 1930] indicated that a small amount was destroyed by the total radiation or by wave-lengths between 210 μμ and 280 μμ, and a slight diminution of absorption occurred. The product of irradiation by the unscreened lamp, in which about 3 % was destroyed, showed no antirachitic activity in rats in doses of 8 γ daily. Irradiation with light of wave-lengths

exceeding 275 $\mu\mu$, under conditions in which ergosterol is destroyed to the extent of 50 %, caused no significant alteration of the absorption or of the amount precipitated by digitonin. A trace of a substance of high melting point appeared to be formed.

Derivatives of α-dihydroergosterol. These were prepared from material containing 0·25 % of ergosterol.

The *benzoate*, prepared with excess of benzoyl chloride in pyridine, and recrystallised, had M.P. 192–195°, $[\alpha]_{5461}^{20}$ − 13·6°. The M.P. of the benzoate prepared from 10 mg. of an authentic specimen of α-dihydroergosterol was 188–194°, and a mixture melted at 190–195°. Heyl and Swoap [1930] record M.P. 153–155°, but from their description it is evident that, owing to the use of insufficient benzoyl chloride, their material must have been a mixture of free sterol and benzoate.

The acetate, prepared by boiling with acetic anhydride for 20 minutes, and recrystallised from ethyl acetate, had M.P. 178–180°, $[\alpha]_{5461}^{20}$ − 24·2°. (Windaus and Brunken [1928] give M.P. 180–181°. Heilbron, Johnstone, and Spring [1929] give M.P. 179–180°, $[\alpha]_{5461}^{20}$ − 25·3°.) (Found (micro): C, 81·9; H, 10·9 %. $C_{29}H_{46}O_2$ requires C, 81·6; H, 10·9 %.)

The *p-nitrobenzoate* was prepared by adding a solution of *p*-nitrobenzoyl chloride (0·3 g.) in chloroform (2 cc.) to α-dihydroergosterol (9·1 g.) in pyridine (2 cc.). The reaction mixture was poured into water, chloroform was removed by evaporation, and the solid was collected, washed with hot alcohol, and recrystallised twice from 80 % aqueous pyridine. It separated in lanceolate plates, melting at 202° to a cloudy, anisotropic liquid, clearing point 248°. It separated from other solvents in gelatinous form. (Found (micro): C, 76·9; H, 8·8 %. $C_{34}H_{47}O_4N$ requires C, 76·5; H, 8·9 %.)

The number of ethylenic linkings in the acetate and benzoate was determined by titration with bromine in carbon tetrachloride according to the modification of Kaufmann's method described by Reindel and Niederländer [1929]. (Found: acetate, 1·9, 1·8; benzoate, 1·7. Control analyses: ergosteryl benzoate, 2·4, 2·4; cholesteryl acetate, 1·1.)

Mixtures resembling neosterol. Neosterol, M.P. 164–165°, $[\alpha]_D^{24}$ − 105°, was isolated by Wieland and Asano [1929] from material derived from the mother-liquors of yeast ergosterol, and the benzoate was described as leaflets having M.P. 173–175°, $[\alpha]_D^{24}$ − 50·6°. The sterol gave the same colour reactions as ergosterol, and its absorption in the ultra-violet [Wieland and Gough (with Page), 1930] was almost identical. The fractions of benzoate crystallising in leaflets encountered in the first stages of the separation strongly resembled the description of neosteryl benzoate. One fraction, for example, had M.P. 170–177°, $[\alpha]_{5461}^{20}$ − 60·0°, and another had M.P. 172–176°, $[\alpha]_{5461}^{20}$ − 63·8°. However, recrystallisation of the first from ethyl acetate and twice from acetone gave a product of M.P. 173–185°, $[\alpha]_{5461}^{20}$ − 47·4°, and two recrystallisations of the second from ethylene dichloride at 37° gave a product of M.P. 180·5–185°, $[\alpha]_{5461}^{20}$ − 46·7°. The hydrolysis of these products yielded sterols which, re-

crystallised from alcohol, had, respectively, M.P. 165·5–170°, $[\alpha]_{5461}^{20}$ − 86·9° and M.P. 168–172°, $[\alpha]_{5461}^{20}$ − 91·5°. (Found (micro), in material dried at 130°/10 mm. over P_2O_5: C, 83·3; H, 9·8 %. $C_{34}H_{48}O_2$ requires C, 83·6; H, 9·8 %.) The antirachitic potency after irradiation was in each case half that of ergosterol, and the extinction coefficients of the alcoholic solution at 281·7, 271·5, and 247·3 μμ were 55–57 % of those of ergosterol. In addition, colorimetric measurement of the second specimen (described below) indicated the presence of about 55 % of ergosterol.

It was thus evident that the material resembling neosteryl benzoate was, in fact, an isomorphous mixture, and this was confirmed, after the recognition of α-dihydroergosterol as one of the constituents, by the preparation of a homogeneous mixed benzoate of ergosterol and α-dihydroergosterol. Ergosterol (0·7 g.) and α-dihydroergosterol (0·3 g.) were dissolved in pyridine (10 cc.) and treated with benzoyl chloride (2·5 cc.). The crude product, separated by pouring into water and washing with alcohol, had M.P. 167–174°, $[\alpha]_{5461}^{20}$ − 65·4°. It crystallised from ethyl acetate in leaflets, M.P. 170–175°, $[\alpha]_{5461}^{20}$ − 62·5°. This entirely confirms the conclusions as to the composition of the early fractions of benzoates, and lends additional support to the suggestion that neosteryl benzoate is also such a mixture.

Colorimetric determination of ergosterol. The blue colour obtained when a chloroform solution of ergosterol or ergosteryl benzoate is mixed with a 90 % aqueous solution of trichloroacetic acid [Rosenheim, 1929] attains a maximum intensity after 5 minutes, and is then readily matched by a combination of blue and yellow Lovibond tints in the Rosenheim-Schuster colorimeter [Rosenheim and Schuster, 1927]. The intensity of the blue is maximal when the reagent and the chloroform solution are mixed in the proportion 2:1, and is then approximately proportional to the concentration of ergosterol, thus providing a rapid method for the determination of ergosterol or the benzoate. Example: a 0·2 % solution of a sterol mixture gave 8·1 blue units (+ 2·7 yellow) in a 10 mm. cell, whilst a 0·1 % solution of ergosterol gave 7·5 blue units (+ 3·2 yellow); the mixture therefore contained 0·1/0·2 × 8·1/7·5 × 100 = 55 % of ergosterol, a result which was in agreement with that of spectroscopic analysis.

Summary.

1. Ordinary yeast ergosterol commonly contains considerable amounts of α-dihydroergosterol.

2. α-Dihydroergosteryl benzoate has been isolated by fractionation of the benzoates from yeast ergosterol, followed by a process of partial bromination which destroys the ergosterol preferentially. α-Dihydroergosterol has been identified and characterised by the preparation of derivatives.

3. The neosterol of Wieland and Asano [1929] has not been found, but the benzoates of ergosterol and α-dihydroergosterol, as well as the free sterols, form mixed crystals whose properties agree with those of neosteryl benzoate and neosterol, respectively.

4. The coloration given by ergosterol with trichloroacetic acid has been applied to its determination.

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XIV. SOME PECULIARITIES IN THE GLYCERIDE STRUCTURE OF LAUREL FATS.

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The broad differences between the types of synthesis that occur in three important sources of natural fats, the seed kernel, the fruit pulp and the animal tissues, were discussed in a previous communication [Collin and Hilditch, 1929], where it was suggested that, whilst the syntheses in the fruit pulp and animal tissues were very similar as far as the manner in which the available fatty acids were arranged as glycerides, the kernel synthesis evidently proceeded in a rather different way, and kernel fats were thereby endowed with a characteristic structure. It was inferred from a consideration of the association ratios (the measure of the degree of dispersion of the unsaturated acid throughout the fat) of the various kernel fats studied, that their structure depends essentially upon an unusually thorough dispersion of the fatty acids among the completed glyceride molecules. Evidence of such a structure was given by all the kernel fats examined with the exception of a sample of the commercially expressed oil of laurel (*Laurus nobilis*). Although this sample was expected to contain a certain amount of pulp fat and therefore to show some irregularity, the structure it actually disclosed was so anomalous that specimens of the true laurel kernel and laurel pulp fat from *Laurus nobilis* berries were prepared in these laboratories and analysed by similar methods for further and more reliable information.

The suggestion in the earlier paper [1929, p. 1278], that the observed abnormality might be due to admixed pulp fat, must be withdrawn for it now appears that the pulp fat is a perfectly normal specimen of its class, and that the anomaly lies entirely in the kernel fat, where the expected even mixed glyceride structure is absent and is replaced by one of extreme heterogeneity.

According to the association ratios, which are approximately constant throughout the kernel fat group, the persistent saturated-unsaturated glyceride mixture in a normal kernel fat contains about 1·4 molecules of saturated acid associated with every molecule of unsaturated acid; and, apparently, when the normal synthesis is taking place, no fully saturated glycerides are formed until this ratio is exceeded in the original mixture of acids. When the proportion of saturated acids in the mixture exceeds this ratio, formation of

saturated-unsaturated glycerides with an average association ratio of 1·4:1 continues until all the unsaturated acids are accounted for and the surplus saturated acids appear as fully saturated glycerides.

The mixture of fatty acids in the laurel kernel fat now studied had the following composition (excluding unsaponifiable): lauric 43·1 %, palmitic 6·2 %, oleic 32·3 %, linoleic 18·4 %. The molecular ratio of saturated to unsaturated acid in this mixture is very near the critical value of 1·4:1 and hence is ideally adjusted for complete conversion into mixed saturated-unsaturated glycerides with formation of no, or at the most of insignificant amounts of, fully saturated material. Actually, however, the kernel fat contains about 36 % of saturated glycerides, and here obviously the more usual mechanism of kernel fat synthesis has not been in operation. The composition of the fatty acid mixture from the saturated glycerides (lauric 95·5 %, palmitic 4·5 %) shows, moreover, that trilaurin is the predominant constituent to the extent of about 90 % of the whole; and it may be recalled that Bömer and Ebach [1928] were also able to isolate about 30 % of trilaurin from laurel oil by direct crystallisation. It should also be emphasised, in view of what follows, that although the saturated portion of the fat is so rich in lauric glycerides the remainder contains only about a quarter of the total lauric acid present in the fat.

A further difficulty is provided by the behaviour of the unsaturated acids. The limiting values for the content of tri-unsaturated glycerides can be calculated from the association ratio of the mixed saturated-unsaturated (and tri-unsaturated) glycerides and must lie between 6 and 31 %. If the heterogeneity observed in the saturated acids extended to the unsaturated acids, as was expected, then the true value would approach the higher limit. In attempting to trace such a correspondence, a part of the fat was hydrogenated as completely as possible (thus transforming all the tri-unsaturated constituents to tristearin) and fractionally crystallised from ether. Although such a method is not strictly quantitative, tristearin is so insoluble in cold ether that it may be isolated fairly completely; in this case the amount of tristearin found was small enough to justify the conclusion that the true value for the tri-unsaturated content approaches the minimum. Thus the unsaturated acids show no tendency to link up with each other after the example of the saturated acids, but distribute themselves in the characteristic way among the residual palmitic and lauric acids left over from the abnormal saturated glyceride formation.

It is difficult to reconcile the behaviour of the two groups of acids without concluding either that they were in different parts of the seed at the time of their conversion into glycerides, or that they arrived in the seed over different periods. The latter suggestion not only covers the behaviour of the acids but also offers some explanation of the peculiar glyceride structure of the fat; for, assuming that the bulk of the lauric acid appears alone in the kernel during the early stages of ripening, then trilaurin alone would be synthesised until,

as the ripening proceeded, the appearance of the unsaturated acids and the rest of the lauric acid would allow the kernel to resume the normal mixed glyceride synthesis, without however affecting the trilaurin already formed. Palmitic, the other saturated acid, would appear from the experimental data to have spread itself over the whole process, reaching its greatest concentration in the second stage of the synthesis along with the unsaturated acids.

A stepwise construction of the fat on these lines would therefore readily account for its structural divergence from the ordinary kernel fat.

Experimental.

The outer pulp was removed without difficulty from the laurel berries leaving a cleanly separated kernel; both pulp and kernels were steeped in acetone to dry and to remove excess fat, ground to a meal, extracted with ether in a Soxhlet and the fat combined with the acetone extract. The berries yielded 70 % kernel (fat content 14 %) and 30 % pulp (fat content 37 %); or, calculated on the original weight of berries, 10·1 % kernel fat and 11·2 % pulp fat.

The kernel fat was solid and light brown in colour. The pulp fat was a dark green liquid, which on long standing deposited small nodules of solid. They possessed the following characteristics:

	Sap. equiv.	Iodine value
Kernel fat	257·4	84·0
Pulp fat	285·7	113·1

The preliminary treatment of each fat followed the same course. They were saponified with 10 % alcoholic potash for 6 hours and the liberated acids separated into "liquid" and "solid" acids by a modification of the Twitchell process. In each case, also, before esterification the "solid" and "liquid" acids were reconverted to soap by the addition of excess of alcoholic potash and the unsaponifiable material removed by repeated extraction with ether.

	"Solid" acids	Non-sap.	"Liquid" acids	Non-sap.
	g.	g.	g.	g.
Kernel fat	32·5	—	39·3	20·2
Pulp fat	26·0	0·6	55·1	7·7

The methyl esters of the purified "solid" and "liquid" acids were prepared and fractionally distilled in the usual manner, and the analytical data thus obtained are summarised below.

Laurel kernel fat.

	"Solid" acids (35·3 %)	"Liquid" acids (64·7 %)	% of fatty acids: Total	% of fatty acids: Excluding non-fatty material
Lauric	25·0	8·2	33·2	43·1
Palmitic	4·8	—	4·8	6·2
Oleic	5·5	19·4	24·9	32·3
Linoleic	—	14·2	14·2	18·4
Unsaponifiable	—	0·9	22·9	—
„ (extracted before methylation)	—	22·0		

Molecular ratio of saturated to unsaturated acids 1·33 : 1.

Laurel pulp fat.

	"Solid" acids (29·8 %)	"Liquid" acids (70·2 %)	% of fatty acids: Total	% of fatty acids: Excluding non-fatty material
Lauric	1·3	1·1	2·4	2·7
Palmitic	18·3	—	18·3	20·3
Oleic	8·0	48·9	56·9	63·0
Linoleic	1·5	11·1	12·6	14·0
Unsaponifiable	—	0·5	9·8	—
„ (extracted before methylation)	0·7	8·6		

Molecular ratio of saturated to unsaturated acids 0·34 : 1.

Estimation of the fully saturated glycerides.

This was carried out in the usual way by treating a solution of the fat in acetone with powdered potassium permanganate and isolating the unchanged saturated glycerides from the oxidation mixture by washing with ammonia and potassium carbonate.

Laurel kernel fat.

150 g. of the kernel fat gave 42·0 g. of crude fully saturated glycerides (iodine value 1–2), and these, on further purification by boiling with aqueous potassium carbonate yielded:

(*a*) 31·9 g. (corr. wt.) neutral fat, sap. equiv. 211·1 (acid value 0·4);

(*b*) 3·2 g. (corr. wt.) fat extracted by ether, sap. equiv. 231·2 (acid value 6·2);

(*c*) 6·9 g. (corr. wt.) mainly acidic material, sap. equiv. 199·2 (acid value 45·7).

Assuming that the acidic matter present in (*b*) and (*c*) is azelaodilaurin (acid value 89·5), the proportion of fully saturated glycerides in the original material is 26·6 %; allowing for the 22 % of non-fatty compounds present in the original, the proportion of fully saturated glycerides in the true kernel fat is therefore 34·2 %.

The fully saturated portion (*a*) was converted into methyl esters, which gave the following results on fractionation:

g.	B.P./1 mm.	Sap. equiv.
4·28	73–93°	210·2
5·66	93	213·2
7·62	93	213·4
3·36	Residue	251·4*

* Residual esters, freed from unsaponifiable matter, sap. equiv. 228·0.

This corresponds with a composition of lauric acid 94·1 %, palmitic acid 4·3 % and unsaponifiable matter 1·6 %, or, excluding unsaponifiable matter, lauric acid 95·5 % and palmitic acid 4·5 %.

The general composition of 100 parts of the original kernel fat, based on the preceding data, is as follows:

	Total fat	Fully saturated glycerides	Mixed saturated-unsaturated or tri-unsaturated glycerides (by difference)	
	100 g.	26·6 g.	73·4 g.	
Unsaponifiable	22·0	0·4	21·6	
Glyceryl residue	3·9	1·5	2·4	
				Molecular ratios
Lauric	31·9	23·6	8·3	415
Palmitic	4·6	1·1	3·5	135
Oleic	23·9	—	23·9	848
Linoleic	13·7	—	13·7	489

The preponderance of lauric acid in the fully saturated portion and of palmitic acid in the rest of the fat may be noted. From the figures in the final column it appears that the "association ratio" in the mixed glycerides is about 0·4 mol. saturated acids per mol. of unsaturated acids. Consequently, of 100 parts by weight of the original kernel fat (containing 22 % non-fatty matter), 26·6 parts consist of fully saturated glycerides as described, whilst about 6–31 parts are tri-unsaturated glycerides, according as the amount of mono-unsaturated-disaturated glycerides lies between nil and 20, and that of di-unsaturated-monosaturated glycerides between 45 and nil.

Tristearin content of the hydrogenated fat. The original kernel fat (40 g.) was hydrogenated repeatedly with 5 g. nickel catalyst until the iodine value fell to 14. Great difficulty was experienced in attaining even this degree of saturation, probably because the large amount of non-fatty matter was either toxic to the catalyst or was itself difficult to hydrogenate; the residual iodine value is almost certainly to be attributed to the non-fatty constituents. The tri-unsaturated glycerides being thus converted to tristearin, the mixture (39·4 g.) was fractionally crystallised from ether. From the first crystallisation of the hydrogenated fat the following fractions were obtained by successive concentrations of the filtrate from each crop of crystals:

Crop	g.	M.P.
A	5·7	64–65°
B	4·8	49–50°
C	6·1	41–43°
D	9·4	33·5–35°
E	13·4	— (*ex* final mother-liquors)

On recrystallisation of crop A, a first fraction (2·8 g., M.P. 66·5–67·5°, sap. equiv. 291·2) was obtained, whilst the more soluble part melted at about 64–65° and had a sap. equiv. of 284·0. Recrystallisation of crop B yielded only a minute amount of material melting at 64–65°, the residue having a maximum melting point of about 52–53°. Since even the least-soluble and highest-melting fraction from crop A is not entirely tristearin, it is quite clear from this examination that the proportion of tri-unsaturated glycerides in the original fat cannot greatly have exceeded the minimum amount shown to be present by the previous quantitative oxidation experiments.

Laurel pulp fat.

100·5 g. of the pulp fat yielded 2·7 g. of saturated material (sap. equiv. 267·5). This corresponds with a fully saturated glyceride content of approximately 3 % on the glycerides present in the pulp fat.

The saturated compounds gave on hydrolysis fatty acids which, after removal of unsaponifiable matter, possessed an equivalent of 255·6 and a melting-point of 60–62° (unaltered on admixture with pure palmitic acid); the glyceride present was thus substantially tripalmitin.

The proportion of fully saturated glycerides, although small, is thus quite well defined, and it is interesting to observe that it is coincident with that of the corresponding synthetic triglyceride mixture as indicated by the graph correlating the proportion of fully saturated glycerides with the proportions of saturated and unsaturated acids in the mixed fatty acids of a synthetic triglyceride [Bhattacharya and Hilditch, 1930]. In common with other fruit pulp fats and with animal tissue fats, therefore, the glyceride structure of laurel pulp fat is closely similar to that of the corresponding synthetic mixed triglycerides.

In conclusion the author wishes to thank Professor T. P. Hilditch for his advice and encouragement during the investigation.

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XV. IODIMETRIC DETERMINATION OF REDUCING SUGARS IN THE APPLE.

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It was pointed out by Judd [1920] that the polarimetric method in combination with the copper reduction method for the determination of fructose and glucose in mixtures of these sugars was unsuitable for the analysis of apple extracts, not only because of the low optical rotation of the extracts, but because they are always coloured and frequently viscous. Evans [1928] investigated various methods of clearing the extracts and found that considerable dilution was necessary for satisfactory clarification. In order to obtain a sufficiently large reading on the polarimeter the cleared solutions had to be evaporated under reduced pressure to a suitable concentration, making the analysis very tedious. Both these workers recommended the use of the iodimetric method [Baker and Hulton, 1920; Cajori, 1922; Willstätter and Schüdel, 1918] in combination with copper reduction for the estimation of fructose and glucose in the apple. This method is based on the oxidation of the sugars by alkaline iodine, and some disagreement existed as to the extent of the oxidation until Hinton and Macara [1924] reinvestigated the reaction. They established the fact that glucose was quantitatively oxidised to gluconic acid in 10 minutes at room temperature, provided not more than half the iodine added was reduced and the ratio of iodine to sodium hydroxide in terms of normal concentration was 1:1·25. They also found that fructose was slightly oxidised at 17·5°, the amount of oxidation varying with concentration, amount of alkali present, time of reaction and temperature. Hinton and Macara attribute the discrepant results obtained by earlier workers to the use of impure samples of sugars.

Evans [1928] repeated Hinton and Macara's work on the oxidation of glucose at 17·5° and also made observations at 5°. He found that oxidation of glucose was complete in 45 minutes at 5° and obtained the same iodine value at both temperatures, namely 1·402 g. of iodine reduced per g. glucose. This is slightly lower than the theoretical value 1·410 obtained by Hinton and Macara. Evans obtained somewhat higher values than Hinton and Macara for the amounts of iodine reduced per g. fructose in spite of the lower

temperature, probably owing to the much longer time of reaction. From the oxidation of invert sugar by iodine at 5° Evans found 0·022 g. of iodine reduced per g. of fructose. Hinton and Macara determined the iodine value for fructose in solutions containing large amounts of fructose relative to glucose and deduced from their results that in invert sugar the value would be 0·012 g.

From the results obtained by these observers it seemed clear that before applying the iodimetric method to apple extracts a thorough investigation of the oxidation of fructose under carefully controlled experimental conditions was necessary, since by far the largest proportion of reducing sugar in the apple is fructose. Examination of the methods of purification of glucose used by Hinton and Macara and by Evans showed that the latter did not employ a high temperature for the final drying of the sample of glucose used. As it is difficult to remove the last traces of water from the sugars without applying heat it is possible that the slightly low values Evans obtained for the amount of iodine reduced per g. of glucose were due to insufficient drying. Since the oxidation of fructose by iodine is considerably affected by both temperature and time of reaction, it was thought that more consistent results would be obtained by using the longer time of reaction necessary for complete oxidation of glucose at a low temperature than by using the short time necessary at the temperature employed by Hinton and Macara. Small variations in either time or temperature would result in less difference in the amount of oxidation of fructose at a low than at a high temperature; for this reason Evans worked at a temperature of 5°. The oxidation by alkaline iodine of carefully purified samples of glucose and fructose has now been investigated at 1°.

It has also been necessary to study the effect of clearing and of the use of different deleading agents on the results obtained by the iodimetric method. In the preparation of apple extracts for estimation of sugars by the copper reducing method Haynes and Archbold [1928] found basic lead acetate and potassium oxalate satisfactory as clearing agents, although a correction of 3 % of the sugar estimated was necessary owing to the loss of sugar in the clearing process. They also found that reliable results could be obtained by using the uncleared solution, obtained by diluting either the expressed juice or an alcoholic extract after evaporation of the alcohol.

Preparation of samples of glucose and fructose.

A sample of glucose purchased as pure was recrystallised three times from alcohol and water and then dried *in vacuo* over phosphorus pentoxide for 4 months. It was then dried at 105° to constant weight (2–3 hours). An ash determination was carried out, and the rotatory power and copper reducing power of the sample found. The results were as follows:

$[\alpha]_D^{20^\circ}$ (10 % solution) observed 52·73°; calculated 52·74° Ash < 0·01 %

Copper reducing power. Weight of glucose taken 0·4056 g./100 cc.
Weight of glucose found 0·4055 g./100 cc.

Fructose was prepared by the method of Harding [1922] used by Hinton and Macara and by Evans. It was found necessary to use 300 cc. of 75 % alcohol to dissolve 400 g. of sugar instead of 200 cc. stated by Harding. The sample was recrystallised three times and then dried first over sulphuric acid and then over phosphorus pentoxide to constant weight. It was then further dried at 50° till again constant. The rotatory power was then observed at 27° and the copper reducing power determined.

$[\alpha]_D^{27°}$ (5·59 % solution) observed $-88·19°$; calculated (using Vosburgh's [1920] formula) $-88·15°$.

Copper reducing power. Weight of fructose taken 5·59 g./100 cc.
Weight of fructose found 5·52 g./100 cc.

Details of method for determining the iodine value of sugar solutions at 1°.

The procedure advocated by Hinton and Macara [1924] was adopted except that the reaction was allowed to proceed at 1° instead of at 17·5°. In order to keep the reaction mixture at the correct temperature the stock solutions of iodine and alkali were kept in a refrigerator at 1°.

Measured amounts of the sugar solution containing about 0·08 g. of sugar were run into glass bottles and the solutions made up to 100 cc. The bottles were stoppered and placed at 1°; when the solutions had cooled down to this temperature 20 cc. of 0·1 *N* iodine and 5 cc. of 0·5 *N* sodium hydroxide were added. The bottles were then immersed in a water-bath at 1° and rotated slowly during the progress of the reaction. Blank determinations were carried out omitting the sugar solution and adding instead 100 cc. of water. These determinations always agreed with the values obtained by direct titration of iodine (measured at 1°) and thiosulphate. When the reaction was complete the solutions were immediately acidified with 5 cc. of 2 *N* sulphuric acid and the excess iodine titrated with standard sodium thiosulphate (0·05 *N*), using starch as indicator. The starch solution was made up with saturated sodium chloride as first suggested by Hagedorn and Jensen [1923]: 130 g. of sodium chloride were dissolved in 400 cc. of boiling water and 5 g. of soluble starch, mixed to a paste with cold water, added to the boiling salt solution; the mixture was boiled for 5 minutes and then allowed to cool and diluted to 500 cc. This solution keeps indefinitely.

Effect of varying the time of reaction on the oxidation of glucose at 1°.

In order to determine the time necessary for the complete oxidation of glucose to gluconic acid at 1° the alkaline iodine was allowed to react with equal amounts of a glucose solution for various times. The results are shown in Table I. The reaction was complete after 2 hours and the theoretical value 1·410 g. of iodine reduced per g. of glucose was obtained. No appreciable difference in this value was found if the reaction was allowed to continue several hours longer, but after prolonged standing (17 hours) a slight increase in the iodine value was observed. There is therefore probably a slow secondary

Table I. *Effect of time of reaction on the oxidation of glucose by alkaline iodine at 1°.*

Amount of glucose present in each estimation = 0·0811 g.
Blank titration 38·05 cc. of 0·051 *N* sodium thiosulphate = 20 cc. iodine (0·1 *N* approx.).

Time of reaction in hours	cc. thiosulphate = iodine reduced	g. iodine reduced per g. glucose
1	17·55	1·395
1½	17·70	1·408
2	17·75	1·411
2½	17·75	1·411
3	17·70	1·408
4	17·75	1·411
5	17·70	1·408
6	17·75	1·411
7	17·75	1·411
17½	17·80	1·415

oxidation of glucose by alkaline iodine, but for many hours the amount is too small to be detected.

The iodine value for glucose, 1·410 g., was confirmed by further estimations on different amounts of glucose, the time of reaction being 2 hours. One set of results is shown in Table II. Hinton and Macara's result for glucose is thus

Table II. *Oxidation of glucose by alkaline iodine at 1°.*

Blank titration 38·05 cc. of 0·051 *N* sodium thiosulphate = 20 cc. iodine.

	Sample I Weight of glucose in each estimation = 0·0643 g.			Sample II Weight of glucose in each estimation = 0·0794 g.	
No.	Thiosulphate = iodine reduced cc.	Iodine per g. glucose g.	No.	Thiosulphate = iodine reduced cc.	Iodine per g. glucose g.
1	14·05	1·412	1	17·35	1·409
2	14·10	1·416	2	17·35	1·409
3	14·10	1·416	3	17·35	1·409
4	14·05	1·412			
5	14·00	1·407	Mean of 8 estimations 1·411.		

confirmed for a temperature of 1°, and it seems probable that, as suggested above, Evans's sample of glucose was not quite dry.

The oxidation of fructose at 1° in solutions containing different ratios of fructose to glucose.

The ratio of fructose to glucose in the mature apple varies in general from about 2·5/1 to 4·5/1, but in a few cases ratios as high as 6/1 may occur. Having established the iodine value for glucose at 1° therefore, a series of estimations was carried out on solutions containing proportions of fructose to glucose ranging from 0/1 to 40/1 in order to determine the correct iodine value of fructose in the presence of glucose.

For this purpose a 0·3 % solution of glucose and a 4 % solution of fructose were prepared. 20 cc. of the glucose solution were taken for each estimation and quantities of fructose varying from 8 to 40 cc. All volumes were made

up to 100 cc., and the estimations carried out as described above. Two or more determinations were carried out for each ratio of fructose to glucose. The amount of iodine reduced by fructose was found by subtracting the amount reduced by glucose alone from the total amount reduced by a given solution. The results are shown in Table III. It will be seen that the iodine value for

Table III. *Oxidation of fructose by alkaline iodine at 1° in mixtures of glucose and fructose.*

Time of reaction 2 hours.
cc. of 0·5 *N* NaOH = 5.
Weight of glucose in each estimation = 0·06 g. approx.
Titration of glucose alone = 29·75 cc. of 0·043 *N* thiosulphate.

Ratio fructose glucose (approx.)	Fructose present g.	Thiosulphate = iodine reduced by fructose cc.	Iodine reduced per g. of fructose g.
1 : 1	0·0617	0·15	0·0134
2 : 1	0·1234	0·35	0·0157
3 : 1	0·1850	0·55	0·0163
4 : 1	0·2454	0·75	0·0169
5 : 1	0·3085	0·95	0·0170
6 : 1	0·3927	1·15	0·0162
7 : 1	0·4250	1·20	0·0156
8 : 1	0·4980	1·30	0·0144
9·5 : 1	0·5750	1·45	0·0139
11 : 1	0·6900	1·70	0·0136
24 : 1	1·5671	3·00	0·0106
30 : 1	1·9589	3·60	0·0102
40 : 1	2·7020	4·70	0·0096

fructose increases from 0·013 g. to 0·017 g. as the ratio of fructose to glucose increases from 1/1 to 5/1; and then decreases again slowly reaching a value of 0·0096 when the ratio is 40/1. Over the range of fructose/glucose ratios found in the apple the value is 0·016 to 0·017; 0·017 being the value for ratios 4/1 and 5/1. Since a difference of 0·001 in the iodine value of fructose makes only a negligible difference in the calculation of the amounts of fructose and glucose in their mixtures, the value 0·017 has been adopted for the whole range of fructose/glucose ratios in the apple. The value for equal amounts of the two sugars under these experimental conditions is 0·013 g., nearly the same as that suggested by Hinton and Macara for a temperature of 17·5° and rather lower than that found by Evans at 5°.

Effect of variations in time of reaction and proportion of alkali on the iodine value of a mixture of glucose and fructose at 1°.

Variations in the iodine value of fructose in the presence of glucose with alkali concentration and time of reaction were next studied, to define the precision necessary to obtain accurate results at 1°. Hinton and Macara showed that if the alkali exceeded the amount required for conversion of the iodine to hypoiodite and iodide, slightly low results were obtained for the iodine value of glucose at 17·5°. In Table V the small effect of excess alkali on the glucose has been neglected, so that the time change in amount of iodine

reduced by fructose is probably slightly greater than the recorded value. It will be seen in Tables IV and V that even at the low temperature of 1° the oxidation of fructose is markedly affected by differences in both amount of alkali and time of reaction.

It is thus essential not to exceed the time necessary for complete oxidation of glucose to gluconic acid by more than a few minutes before stopping the reaction, and also to regulate the alkali concentration so that the amount present is just sufficient to convert the iodine to hypoiodite. The results are shown in Tables IV and V.

Table IV. *Effect of variation in the time of reaction on the iodine value of fructose at 1°.*

Ratio fructose/glucose = 2·5/1.
Amount of glucose in each estimation = 0·06 g. (approx.).
Titration of glucose alone = 27·00 cc. of 0·044 *N* sodium thiosulphate.

Time in hours	Thiosulphate = iodine reduced by fructose (cc.)	Iodine reduced per g. fructose (g.)
2	0·50	0·0179
2½	0·60	0·0215
3	0·65	0·0232
5	0·90	0·0321
6	1·00	0·0358
17	1·60	0·0573

Table V. *Effect of varying the proportion of alkali on the iodine value of fructose at 1°.*

Ratio fructose/glucose = 2·5/1.
Amount of glucose in each estimation = 0·06 g. (approx.).
Blank titration 44·20 cc. of 0·044 *N* sodium thiosulphate = 20 cc. iodine.
Titration of glucose alone = 26·60 cc. of 0·044 *N* sodium thiosulphate.

cc. 0·5 *N* NaOH added	Thiosulphate = iodine reduced by fructose (cc.)	Iodine reduced per g. of fructose (g.)
4	Insufficient alkali for complete oxidation of glucose present	—
5	0·5	0·0174
6	0·6	0·0209

The application of the iodimetric method to the determination of reducing sugars in apple extracts.

In his work on the sugars in the apple Evans [1928] determined the amounts of fructose and glucose present by combination of the iodimetric method and Lane and Eynon's [1923, 1] method of estimating reducing sugars by copper reduction. The values for the two sugars were obtained by solving the simultaneous equations

$$C_1x - C_2y = \text{iodine value per 100 cc. of solution,}$$

$$K_1x - K_2y = \text{copper reducing power of 100 cc. of solution,}$$

where C_1 and C_2 are the g. of iodine reduced per g. of glucose and fructose respectively, and K_1 and K_2 the g. of cuprous oxide formed per g. of glucose

and fructose at the dilution used. Substitution of the more accurate values 1·410 and 0·017 for the g. of iodine reduced per g. of glucose and fructose respectively for those used by Evans makes a difference of less than 1 % in the amount of fructose, and about 0·0005 % in the amount of glucose calculated by solving the above equations. There is therefore no serious error in the results quoted by him. Solutions obtained by diluting the expressed juice of the apple and clearing with basic lead acetate and potassium oxalate were used for the estimations, and Evans stated that potassium oxalate and sodium acetate are not attacked by alkaline iodine (see p. 109).

The clearing of apple extracts with basic lead acetate and potassium oxalate has since been thoroughly investigated by Haynes and Archbold [1928] who found that in cleared solutions the results of sugar estimations were about 3 % lower than in uncleared solutions. They further showed that a similar loss of sugar occurred in clearing artificial mixtures of sugars, acid and pectin, of the same composition as the apple extract. In the estimation of total and reducing sugars by copper reduction clearing of the apple extracts is thus not necessary. Estimations in the dilute uncleared solution must, however, be carried out immediately, as hydrolysis of sucrose occurs on standing. This is not the case in cleared solutions, which remain unchanged for months—a distinct advantage during a series of routine estimations. A comparison between the iodine values of cleared and uncleared apple juices showed differences amounting to 10 %, the uncleared solutions giving the higher values. The clearing was carried out in dilute solution as described by Evans [1928, p. 4] and the uncleared juice was diluted to the same extent for estimation. 75 to 100 cc. of the dilute solutions (containing about 0·06 g. reducing sugar) were used for each estimation, and the reaction was carried out as described on p. 103, except that 25 cc. of iodine and 6 cc. $N/2$ NaOH were used. The volume of sugar solution was always made up to 100 cc. before adding the iodine and alkali. It should be pointed out that this solution is diluted to twice its volume for the determination of copper reducing power. The iodine values must therefore be halved for calculation of the amounts of fructose and glucose by means of the simultaneous equations. The results are shown in Table VI and point to the presence of substances oxidisable by iodine other than sugar in the uncleared extract, since clearing is known to cause a loss of only 3 % in the sugars.

Table VI. *The iodine values of cleared and uncleared apple juices.*

100 cc. of diluted solution used for each estimation.
Blank titration 50·6 cc. of 0·049 N thiosulphate = 25 cc. iodine.

	Thiosulphate = iodine reduced (cc.)		Iodine reduced by 100 cc. of diluted solutions (g.)	
Sample	Cleared juice	Uncleared juice	Cleared juice	Uncleared juice
1	25·65	28·25	0·1595	0·1757
2	21·00	28·80	0·1306	0·1792
3	25·80	27·90	0·1604	0·1736

Hinton and Macara [1927] have suggested the use of chloramine-T and potassium iodide as a weaker oxidising agent than alkaline iodine, for the determination of aldose sugars in cases where oxidisable material other than sugar is present, but it was found on trial that the results of estimations in cleared and uncleared solutions still showed a difference of 10 %. Hence no advantage is obtained by using chloramine-T instead of alkaline iodine for the determination of fructose and glucose in apples. Clearing is therefore essential if the iodimetric method is to be used satisfactorily, and an advantage if only copper reducing values are required, since it avoids the necessity of immediate estimation. The clearing process has in consequence been re-investigated to determine its effect on the iodine values of sugar solutions.

For this purpose the following solutions were prepared: fructose, 20 %; glucose, 8 %; malic acid, 4 %; pectin, 0·3 %. Equal amounts of the four solutions were mixed, giving a solution of these substances in the concentrations in which they occur in the apple. Mixtures were also made omitting the pectin, and omitting both the pectin and the malic acid, and diluting with water to obtain the appropriate concentrations. Two aliquots of each solution were diluted and neutralised in exactly the same way as the apple juice. One aliquot was cleared with basic lead acetate and potassium oxalate, and the iodine value of 100 cc. determined while the iodine value of the other was determined in 100 cc. of uncleared solution. The results are shown in Table VII. In every case a higher value was obtained in the cleared solution.

Table VII. *The iodine values of sugar solutions before and after clearing with basic lead acetate and potassium oxalate.*

Approximate concentrations of constituents in the diluted solutions used for estimations:

Solution F = Fructose 0·2 %.
„ G = Glucose 0·08 %.
„ M = Malic acid 0·04 %.
„ P = Pectin 0·003 %.

Blank titration 51·35 cc. of 0·049 *N* thiosulphate = 25 cc. iodine.

	Thiosulphate = iodine reduced (cc.)		Iodine reduced by 100 cc. of solution (g.)	
Solution used	Uncleared	Cleared	Uncleared	Cleared
Sample 1				
F + G	17·70	18·08	0·1101	0·1124
F + G + M	17·65	17·90	0·1097	0·1114
F + G + M + P	17·75	18·00	0·1104	0·1120
Sample 2				
F + G + M	17·90	18·48	0·1114	0·1149
F + G + M + P	17·90	18·35	0·1114	0·1141
Sample 3				
F + G + M	16·50	16·80	0·1027	0·1045
F + G + M + P	16·48	16·85	0·1024	0·1050

It has been stated by Davis [1916], Deerr [1916] and Geerligs [1909] that the loss of sugar on clearing with basic lead acetate is chiefly a loss of fructose. In that case no marked decrease in the iodine value, similar to that found in

the copper reduction value, would be expected since the amount of oxidation of fructose is so small. The consistently high results for the iodine values found in the cleared solutions, however, suggest that some oxidation by iodine of the potassium oxalate occurs. Consequently the effect of iodine on potassium oxalate alone and in the presence of glucose was investigated. Varying amounts of oxalate alone were allowed to react with iodine under the normal experimental conditions, while in a second set of estimations the solutions were acidified and titrated immediately. In both cases some oxidation occurred, the differences in titration from the blank varying from 0·1 cc. to 0·8 cc. of 0·05 *N* sodium thiosulphate as the amount of oxalate increased from 0·5 cc. to 10 cc. In the presence of glucose the amount of iodine used was greater than that required for the complete oxidation to gluconic acid by an amount equivalent to 0·2 cc. of 0·05 *N* sodium thiosulphate per cc. of potassium oxalate, a similar amount to that found using oxalate alone. There is therefore a small but definite oxidation of oxalate under these conditions not affected by the presence of sugar, and the use of potassium oxalate as a deleading agent requires a correction of about 0·2 cc. to the titration obtained. Since a correction has also to be made to the values obtained by copper reduction it was decided to try other deleading agents.

The effect of different clearing and deleading agents on the iodine values of sugar solutions.

Solutions of fructose and glucose containing in some cases malic acid and pectin, were cleared with both normal and basic lead acetate and deleaded with potassium oxalate, sodium carbonate and sodium phosphate (Na_2HPO_4). Colloidal ferric hydroxide, as suggested by Evans, was also tested but proved less convenient owing to the bulky nature of the precipitate obtained. The method adopted was similar to that already described for testing the effect of clearing and solutions of the same strengths were employed (see Table VII). With both normal and basic lead acetate potassium oxalate gave higher values for the iodine equivalents in the cleared than in the uncleared solutions. Sodium carbonate did not completely remove the lead from solutions cleared with normal lead acetate, and in the course of the iodimetric estimation a precipitate of lead iodide was obtained on the addition of acid to the reaction mixture [see also Meade and Harris, 1916]. With basic lead acetate as a clearing agent, the removal of lead appeared to be complete, but the filtrate was not clear, and the iodine equivalent was about 1·5 % too low in the cleared solution.

With sodium phosphate (Na_2HPO_4) and normal lead acetate a clear filtrate was obtained, but the solution was acid and required neutralising before the addition of alkaline iodine in order that the correct proportion of alkali should be present during the oxidation reaction. Using basic lead acetate a neutral filtrate was obtained, and the solution could readily be filtered at once. In addition, estimations of sugar, both iodimetrically and by copper reduction

showed that by using sodium phosphate as the deleading agent there was no loss of sugar in the clearing process. Norris and Brodie [1918] state that sodium phosphate completely removes lead and has no effect on the reducing power of sugar solutions, and Englis and Tsang [1922] found the smallest loss of sugar with this deleading agent.

Further trials with sodium phosphate as a deleading agent, using both normal and basic lead acetate as clarifying agents, were then carried out. The sugar solutions containing acid and pectin were cleared and deleaded, and allowed to stand for various times before filtering. It was found that prolonged standing, 3 days, caused re-solution of some lead, since both with normal and basic acetate a precipitate of lead iodide appeared during the iodimetric estimation. The agreement between the results for shorter times of standing up to 16 hours was good in both cases. In view of the extra process of neutralisation required when normal lead acetate is used, and also the incomplete precipitation of malic acid by this salt, it was decided to continue the use of basic lead acetate for clearing the apple extract, but to use sodium phosphate instead of potassium oxalate as a deleading agent. The results of these trials are shown in Tables VIII and IX.

Table VIII. *The effect of clearing with normal and basic lead acetates, and of using different deleading agents on the iodine values of sugar solutions.*

Solutions as in Table VII.
Blank titration 57·4 cc. of 0·044 *N* thiosulphate = 25 cc. iodine.

Clearing agent	Solutions used	Thiosulphate = iodine reduced (cc.)		Iodine reduced per 100 cc. of solution (g.)	
		Uncleared	Cleared	Uncleared	Cleared
	Deleading agent potassium oxalate.				
	Sample 4				
Normal lead acetate	F + G	24·00	24·25	0·1330	0·1343
	F + G + M + P	24·05	24·45	0·1335	0·1354
	Deleading agent sodium carbonate.				
Normal lead acetate	F + G	24·00	25·00	0·1330	0·1385*
	F + G + M + P	24·00	24·25	0·1330	0·1343*
	Sample 5				
Basic lead acetate	F + G + M + P	22·38	22·05	0·1237	0·1222
	Deleading agent sodium phosphate (Na_2HPO_4).				
Basic lead acetate	F + G + M + P	22·38	22·40	0·1237	0·1241
	Cleared with colloidal ferric hydroxide.				
	F + G	22·25	22·75	0·1233	0·1260
	F + G + M + P	22·30	23·00	0·1237	0·1274

* Lead iodide formed during the reaction.

Lane and Eynon [1923, 2] have objected to the use of sodium phosphate as it does not entirely remove alkaline earth metals, and their presence will affect the copper reduction of a sugar solution. This objection, however, does

Table IX. *The effect of time of standing on the iodine values of solutions cleared with normal and basic lead acetate and deleaded with sodium phosphate.*

Blank titration 57·0 cc. of 0·044 *N* thiosulphate = 25 cc. iodine.
Iodine values of 100 cc. of uncleared solutions 0·1327 g.[1]; 0·1282 g.[2]

Time of standing	Thiosulphate = iodine reduced (cc.) Clearing agent		Iodine reduced per 100 cc. solution (g.) Clearing agent	
	Normal lead acetate	Basic lead acetate	Normal lead acetate	Basic lead acetate
Filtered at once	—	23·90	—	0·1324[1]
2 hours	23·05	23·10	0·1277	0·1280[2]
Overnight	23·20	23·15	0·1285	0·1282[2]
3 days	Lead iodide formed			

not hold for apple extracts since the amounts of calcium present are too small to cause any error.

The use of sodium phosphate as a deleading agent in the clearing of apple extracts.

As the use of basic lead acetate and sodium phosphate had proved quite satisfactory for clarification of mixtures of sugars, malic acid and pectin, this method of clearing was applied to a series of extracts from stored apples. These extracts were prepared both by alcoholic extraction of the fresh tissue [Haynes and Archbold, 1928, p. 976] and by expression of the juice from previously frozen pulp [Haynes, 1925, p. 78]. The clearing method described by Evans was used, but a saturated solution of sodium phosphate was substituted for the potassium oxalate. 2–3 cc. of phosphate were added for each cc. of basic lead acetate solution. The solutions prepared from the alcoholic extractions were found to develop a yellow colour on standing, the colour becoming more intense as the time of standing was prolonged. No improvement was obtained by keeping in the dark instead of exposed to direct sunlight on the bench. The solutions prepared from the expressed juice remained quite colourless on standing. No colour appeared in either case if the solutions were deleaded with potassium oxalate. A comparison of the iodine values showed that the values for the coloured solutions which had been deleaded with sodium phosphate were considerably higher than those for the colourless solutions deleaded with potassium oxalate. Three sets of comparative values are shown in Table X. No correction has been made for the iodine reduced by the potassium oxalate; this correction would of course slightly increase the difference between the two sets of observations. Extracts prepared from very immature fruit were found to be coloured whichever deleading agent was used, and by solving the equations obtained by determining the iodine value and copper reducing power, negative values for fructose were obtained. These results show that in the coloured solutions iodine is reduced by some substance other than sugar.

Table X. *Iodine values of solutions prepared from alcoholic extracts of apple tissue cleared with basic lead acetate and deleaded with sodium phosphate and potassium oxalate.*

	Solution deleaded with	
	Sodium phosphate	Potassium oxalate
Solution	Iodine reduced per 100 cc. (g.)	Iodine reduced per 100 cc. (g.)
1	0·1635	0·1513
2	0·1927	0·1722
3	0·1624	0·1465

Since the use of alcoholic extracts is preferable to the use of expressed juice [Haynes and Archbold, 1929] for sugar estimations, and the results obtained when sodium phosphate is used as the deleading agent require no correction for loss of sugars during clearing, the coloured solutions were boiled with charcoal to see if decoloration without loss of sugar was possible.

Table XI. *Iodine values of solutions prepared from alcoholic extracts of apple tissue cleared with basic lead acetate and sodium phosphate and decolorised by charcoal.*

	Coloured solution	Decolorised solution
Solution	Iodine reduced per 100 cc. (g.)	Iodine reduced per 100 cc. of original solution (g.)
1	0·1778	0·1566
2	0·1965	0·1621
3	0·1637	0·1491

Trials were made with animal charcoal and a sample of "suchar[1]." The "suchar" was found to be much more efficient for decolorising the solutions and was used for all subsequent work.

The iodine values of the colourless solutions obtained by boiling with charcoal were next determined. 200 cc. of the neutral cleared, coloured solution were boiled with 0·5 g. of charcoal for 1 minute. The solution was filtered and the charcoal thoroughly washed with boiling water. The volume of filtrate was then made up to 250 cc., and the iodine value determined in the usual way. In every case a decrease in the iodine value was found as compared with the coloured solutions. Three sets of results are shown in Table XI.

In some cases one boiling was not sufficient to decolorise the solution, if this was so the solution was filtered and again boiled for 1 minute with fresh charcoal. This process was repeated until the solution was colourless. The iodine value decreased with each successive boiling until the solution was colourless, after which further boilings had no effect. It was found necessary to boil once more after the solution was apparently colourless in order to ensure a constant result. The effects of increasing the quantity of charcoal used and the time of boiling were tried, but no advantage was obtained in

[1] Suchar is a charcoal specially prepared for sugar refining by the British Suchar Processes, Ltd., and kindly supplied by them.

either case. The results are shown in Table XII. Tests were then carried out with invert sugar solutions to which sucrose had been added to see if adsorption occurred during the boiling process. No change was found in the iodine

Table XII. *Iodine values of a cleared extract of apple tissue after different times of boiling with varying amounts of charcoal.*

No. of boilings	Time of each boiling (mins.)	Wt of charcoal (g.)	Iodine reduced per 100 cc. of original solution (g.)
4 (colourless after 3rd boiling)	1	0·5	0·302
4 (left to stand ½ hour before filtering off charcoal)	1	0·5	0·298
3	1	1·0	0·301
3 (colourless after 2nd boiling)	5	0·5	0·299

value after boiling once with charcoal, and after six boilings a loss of only 0·8 % was observed. The reducing power estimated by copper reduction however decreased 1 % after one boiling and 5 % after six boilings. This loss of sugar occurred in both total and reducing sugars and suggests that a slight adsorption of fructose and sucrose occurs during the boiling, while glucose is not affected. Consequently no change is found in the iodine value after one boiling with charcoal.

The iodine value and reducing sugars determined by copper reduction are shown in Table XIII.

Table XIII. *The effect of boiling with charcoal on the reducing power and iodine values of an invert sugar solution containing sucrose.*

No. of boilings	Reducing sugar (by copper reduction) g./100 cc.	Total sugar (by copper reduction) g./100 cc.	Sucrose g./100 cc.	Iodine reduced per 100 cc. (g.)
Unboiled	0·720	1·032	0·312	0·0753
1	0·713	1·026	0·313	0·0753
2	0·711	1·006	0·295	—
6	0·685	0·936	0·251	0·0747

The copper reducing power can be satisfactorily determined in the uncleared, or in the cleared but coloured solution, and the results combined with iodine values determined in the decolorised solution. The loss of reducing power during decoloration is therefore not a serious drawback to the use of sodium phosphate as a deleading agent. Since the cleared solutions darken considerably on standing, the effect of the more intense colour on the iodine value was next examined. An alcoholic extract of apple tissue was prepared and after evaporation of the alcohol the solution was diluted and cleared with basic lead acetate and sodium phosphate. The solution was colourless immediately after filtering, and an estimation of the iodine value was carried out at once, both with and without a preliminary boiling with charcoal. The

cleared solution was allowed to stand and again estimated after intervals of 3 and 8 months. More than one boiling was necessary to decolorise the solutions after long standing.

The results are shown in Table XIV.

Table XIV. *Iodine values of apple extract cleared and deleaded with sodium phosphate estimated after different periods of standing both in coloured and decolorised solutions.*

	Cleared coloured solutions estimated			Cleared decolorised solutions estimated		
	At once	After 3 months	After 8 months	At once	After 3 months	After 8 months
No. of boilings required	—	—	—	1	2	4
Sample 1	0·2089	0·2089	0·2067	0·1796	0·1750	0·1767
Sample 2	—	0·1635	0·1625	0·1442	0·1448	—
Sample 3	0·1927	0·1907	—	0·1703	0·1739	—

No change was found in the iodine values of either the coloured or decolorised solutions after 8 months. Thus depth of colour does not in itself tend to increase the iodine value. In order to test the validity of the figures obtained by this method a comparison was made with iodine values of solutions deleaded with potassium oxalate; since it was possible that the colourless solution obtained in this way might also contain the oxidisable material, estimations were carried out on solutions which had been boiled with charcoal in addition to the ordinary estimation. In this case the iodine value increased considerably during the boiling, and a solution boiled without the addition of charcoal showed an even greater increase. A solution of fructose and basic lead acetate and potassium oxalate showed the same increase in iodine value after boiling with or without the addition of charcoal, while the iodine value of a solution of potassium oxalate alone was unaffected by boiling. This increase appears to be due to some action of the excess of potassium oxalate on the sugars during boiling. Potassium oxalate is therefore unsuitable as a deleading agent for solutions prepared from immature apples. It has already been stated that such solutions are coloured even if deleaded with oxalate, and the above results make it clear that decolorising by boiling is not practicable.

Table XV. *Iodine values, g. iodine reduced per 100 cc., of solutions deleaded with sodium phosphate and potassium oxalate before and after boiling.*

	Solution deleaded with			
	Sodium phosphate		Potassium oxalate*	
Sample	Not boiled	Boiled	Not boiled	Boiled
1	0·1635	0·1442	0·1513	0·1754
2	0·1927	0·1703	0·1722	0·2040
3	0·1624	0·1423	0·1465	0·1703

* No correction has been made for the oxidation of potassium oxalate. The results are therefore about 0·5 % too high.

The parallel experiments with extracts of mature apples (Table XV) using the two deleading agents showed that nearly the same result is obtained in the solution deleaded with sodium phosphate after decoloration as in that deleaded with potassium oxalate but not boiled. By varying the amounts of phosphate added during clearing it was found that the development of colour was largely dependent on the amount of phosphate present. Three solutions were prepared, one containing insufficient phosphate to remove all the lead, one with a slight excess of phosphate and one with a large excess. After standing for 3 months the first solution was still colourless, the second pale yellow and the third deeper yellow. The one containing excess lead was then completely deleaded, and iodine values were determined on all three. Similar values were obtained for the two solutions which had stood with excess of phosphate, but the third gave a high value indicating a conversion of fructose to glucose in the presence of lead.

From the foregoing results it was concluded that for extracts prepared from mature apples satisfactory results could be obtained by using either potassium oxalate or sodium phosphate as the deleading agent, but that oxalate was inadmissible unless the cleared solution was colourless. The use of oxalate also has the disadvantage of requiring corrections both in the iodine and copper reduction values to give the correct results. The use of sodium phosphate requires no such corrections, but is more tedious since the additional process of decoloration is necessary. Since a good deal of work on immature fruit is in progress in this laboratory sodium phosphate has been adopted for routine work.

It has been found that the iodimetric method can be used satisfactorily for the estimation of much smaller amounts of sugar than those used in the work described here. It is now being employed in combination with the Hanes [1929] modification of Hagedorn and Jensen's method of sugar estimation for the determination of the small amounts of sugar in the developing apple. These results will be published shortly.

[*Note*, added 13th Feb.] The polarimetric method is not only tedious but there are indications that it is liable to give untrustworthy results in the case of apple extracts. Evans's [1928] results show quite close agreement between the actual polarimeter readings and those expected from the iodine and copper values of the solutions. Examination of his paper shows that he has assumed that the specific rotation of fructose is independent of concentration. If his data are recalculated using the formula relating specific rotation and concentration of fructose given by Vosburgh [1920, 1921] it is found that the polarimeter readings are uniformly lower than would be expected from the copper and iodine values. We have made observations which confirm this result, and there is thus some indication of the presence of a laevorotatory substance other than sugar in the cleared apple extract.

SUMMARY.

The oxidation of glucose and fructose by alkaline iodine at 1° and methods of preparation of apple extracts for iodimetric estimations have been investigated. Glucose is quantitatively oxidised to gluconic acid in two hours at 1° and some oxidation of fructose also occurs.

In mixtures of glucose and fructose the amount of iodine reduced per g. of fructose increases from 0·013 g. to 0·017 g. as the ratio of fructose to glucose increases from 1/1 to 5/1 and then decreases slowly as this ratio is further increased. The value 0·017 can be used for determination of fructose and glucose in apples by combination of the iodimetric and copper reduction methods, since the ratio of fructose to glucose in the apple is about 4/1.

The presence of oxidisable material other than sugar makes it necessary to clear the apple extracts before carrying out iodimetric determinations. Basic lead acetate with either sodium phosphate (Na_2HPO_4) or potassium oxalate as the deleading agent was found to give satisfactory results with extracts prepared from mature apples.

The loss of sugar during clearing and the slight action of iodine on potassium oxalate make corrections necessary for both the copper reducing value and the iodine value if oxalate is used as the deleading agent. If sodium phosphate is used there is no loss of sugar, but the cleared solution is yellow and still contains some non-sugar substance oxidisable by iodine. The solution can be decolorised by boiling with charcoal and satisfactory results obtained. Copper reduction determinations can be carried out on the yellow solution.

With very immature apples coloured solutions are also obtained when potassium oxalate is used, and these solutions cannot be boiled, as an increase in the iodine value occurs owing to some action of oxalate on the sugars. Sodium phosphate has therefore been adopted as the deleading agent for routine work.

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XVI. BIOLOGICAL OXIDATIONS IN THE SUCCINIC ACID SERIES.

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The oxidation of succinic acid can be accomplished by cells of very diverse types, ranging from the bacteria to those of mammalian muscle and brain. Early work on the course of this oxidation, by Batelli and Stern, Einbeck, and Dakin, showed that succinic acid was oxidised to fumaric acid and that this took up the elements of water to form *l*-malic acid. Hahn, Haarman and Fischbach [1929] demonstrated that in presence of muscle *l*-malic acid was further oxidised to oxaloacetic acid. In the case of bacteria it was clear [Quastel, 1924] that the main course of oxidation of succinic acid was through fumaric acid and pyruvic acid, oxaloacetic acid presumably being an intermediate step. The question, however, arose as to whether fumaric acid was oxidised directly or whether it was necessarily transformed to malic acid prior to oxidation. It was shown with bacteria, using the methylene blue technique [Quastel and Whetham, 1924], that malic acid was not a ready donator of hydrogen, a fact contrary to what would be expected if the oxidation of fumaric acid proceeded through malic acid. The evidence was sufficient to justify the opinion that fumaric acid might be oxidised directly. The discovery, however, that the donating powers of malic acid might be obscured by the accepting powers of the acid—presumably through the formation of fumaric acid which is a ready hydrogen acceptor—vitiated this conclusion, and later on the formulation of the theory of activation [Quastel, 1926] made it appear very unlikely that fumaric acid would be oxidised directly, an activated fumarate molecule, on the theory, tending to take up positive electricity (protons or hydrogen) rather than negative (oxygen). The question, therefore, was left quite open. Most tissues and organisms can bring about a change of fumaric acid to *l*-malic acid and can accomplish the oxidation of the latter acid, but neither of these facts constitutes evidence that the main line of oxidation of fumaric acid lies through *l*-malic acid. In the case of certain bacteria, for instance, fumaric acid gives rise (in presence of ammonium ions) to *l*-aspartic acid, an amino-acid capable of being oxidised by the bacteria in question. But it cannot be assumed from these facts that the main line of oxidation of fumaric acid, with these bacteria, lies through *l*-aspartic acid. The actual finding among the products of oxidation of succinate or fumarate

of any substance which is capable of further oxidation does not constitute evidence that this substance occurs on the main line of oxidation of the succinate or fumarate. Quantitative, in addition to qualitative, evidence is required in order properly to appraise the significance of any such substance.

An attempt is made in this communication to show, from a quantitative standpoint, that the biological oxidation of fumaric acid proceeds largely, if not entirely, through *l*-malic acid. This is done by comparing the rates of oxidation of fumaric and malic acids in presence of various organisms and tissues, by comparing the action of inhibitors on these oxidations and by measuring the malic acid produced from succinic acid. At the same time measurements have been made of the relative rates of oxidation of succinic, fumaric, malic and aspartic acids in presence of various tissues and organisms and of the influence these substances have on each other's oxidation. Results have been obtained showing very clearly how the organisms and tissues differ in significant ways from each other.

Of considerable importance to the problems of respiration is the inhibitory action of malonate on succinate oxidation, first found by the methylene blue method in the case of bacteria [Quastel and Whetham, 1925; Quastel and Wooldridge, 1928]. It will be shown that this inhibitory action applies not only to the oxidation of succinate by bacteria, but to that by muscle and brain tissue. The degree of inhibition varies widely, being greatest with muscle and brain. The reasons for this and the bearing of the results on the views held by Warburg are discussed.

Experimental.

Oxidation rates were measured in the Barcroft differential manometer, all experiments being carried out at 37°. Acids were used in the form of their sodium salts, the p_H of solutions being 7·4. The oxygen uptakes recorded are the net uptakes due to the substrates alone—the control oxygen uptake by the organism or tissue alone being subtracted from the uptake given by organism or tissue in presence of the substrate. On some occasions the same amount of organism or tissue was placed in each cup of the apparatus, the substrate under investigation being placed only in the right hand one. The oxygen uptake in such cases was that due to the substrate alone.

Phosphate buffer solution (M/15) was always present with the tissue, and substrates were present at an initial concentration of M/15. The total volume of solution in each cup of the apparatus was 3 cc.

The rates of oxidation of substrates were linear over the first 2 or 3 hours of the oxidation indicating that the initial concentration (M/15) used was sufficient to saturate the enzymes involved.

The various bacteria were prepared by 48 hours' growth either in tryptic broth or on nutrient agar plates, the organism in the first case being centrifuged and washed twice with 0·85 % saline, and in the second case being scraped off the plates, suspended in saline and then centrifuged and washed;

after washing, a homogeneous suspension of the organism in saline was prepared. 1 cc. of this suspension was used in each experiment. The organism was used as soon as possible after its preparation, its age being of great importance so far as the oxidations of fumarate, malate or aspartate are concerned. When not in use the organism was stored at 0°.

In the cases of muscle and brain, 0·5 g. of the tissue was always used. Muscle, after dissection from the animal, was cooled and minced. Brain was similarly treated, the whole brain in the case of the rabbit being used. With human brain only the grey matter of the cerebral cortex, after careful removal of the membranes and adhering blood vessels, was used.

In Table I in which most of the experimental results are set out, the

Table I. *Uptake of oxygen (at 37°) in mm.³ by organisms and tissues in presence of various substrates and mixtures of substrates, the initial concentration of each being* M/*15.*

Exp.	Organism or tissue	Succinate	Fumarate	*l*-Malate	*l*-Aspartate	Succinate + fumarate	Succinate + *l*-malate	Succinate + *l*-aspartate	Succinate + malonate
1	*B. coli*	1456	698	770	710	1202	1185	1312	1108
2	,,	1372	662	651	—	1064	924	—	—
3	,, (few days old)	440	17	—	—	251	—	—	45
4	*B. acidi lactici*	1210	716	630	475	—	—	—	—
5	,,	1072	—	—	—	842	889	806	—
6	,,	210	5	—	—	123	—	—	37
7	*B. prodigiosus*	487	293	391	495	335	538	591	382
8	*B. proteus*	1818	984	1035	1270	1445	1578	1525	1550
9	,,	552	237	—	—	406	—	—	345
10	*B. pyocyaneus*	470	550	447	532	—	—	—	—
11	,,	429	—	—	—	370	403	533	—
12	,,	497	—	—	—	—	—	—	279
13	*B. alkaligenes*	358	75	155	257	242	287	575	108
14	,,	541	—	—	—	415	479	1362	—
15	,,	720	248	—	—	496	—	—	386
16	*B. subtilis*	443	—	—	352	—	—	1136	—
17	,,	889	715	971	1342	—	—	—	710
18	*M. lysodeikticus*	728	584	416	242	632	718	1160	645
19	Rabbit muscle	302	—	—	92	—	—	544	—
20	,,	900	45	477	400	528	640	1050	137
21	,,	691	40	10	—	405	300	769	0
22	Rabbit brain	445	50	102	0	365	439	385	3
23	,, (few days old)	410	41	21	—	—	—	—	37
24	Human brain (grey matter)	804	9	—	—	690	—	—	108
25	,,	201	47	38	23	—	—	—	—

results of any one experiment are quantitatively comparable with each other, but not with those of a differently numbered experiment. The results given are representative of a large number of experiments.

Relative rates of oxidation of succinic, fumaric, malic and aspartic acids.

The rates of oxidation, as previously stated, are approximately linear over the first 2 or 3 hours. In most of the experiments a period of 2 hours was made the limiting time, and the actual oxygen uptakes of a number of substrates at the end of this period are a measure of the relative rates of oxidation of these substrates.

Now it follows that if fumaric acid can only be oxidised *via* *l*-malic acid, the velocity of oxidation of fumaric acid cannot be greater than that of *l*-malic acid for equivalent saturation concentrations of these substrates. A distinctly higher rate of oxidation for fumarate would indicate a separate line of oxidation.

The experimental results recorded in Table I show, on the whole, that the velocities of oxidation of fumarate and *l*-malate are of the same order, a much higher figure being recorded, however, in the case of a specimen of rabbit muscle for *l*-malate. It is an interesting fact that in certain cases the velocity of oxidation of fumarate exceeds that of *l*-malate. This is notably the case with *M. lysodeikticus* and it has been observed several times with brain tissue. The excess of oxidation, however, is usually small so that although this excess may point to some direct oxidation of fumarate, the effect may also be attributable to a secondary factor.

The fact that the oxidations of fumarate and *l*-malate are usually of the same order points to the rapid transformation of fumarate into *l*-malate and hence to the presence of fumarase in the organisms and tissues investigated. Experiment shows this to be the case.

The oxidation of aspartic acid is of interest. Aspartic acid may undergo (1) a direct oxidation, or (2) loss of ammonia, in presence of those organisms possessing the necessary enzymes, to form fumaric acid [Quastel and Woolf, 1926; Cook and Woolf, 1928] and oxidation *via* *l*-malic acid. If course (2) is the sole mode of oxidation followed, the velocity of oxidation of aspartic acid should not be greater than that of *l*-malic acid; if course (1) takes place the velocity may be greater. The results show that the strict aerobes *B. alkaligenes* and *B. subtilis* oxidise aspartate at a much greater rate than *l*-malate, whereas the facultative anaerobes, *B. coli*, *B. acidi lactici*, oxidise the amino-acid at about the same or a lesser rate.

The results suggest that the strict aerobes in question attack the amino-acid by course (1) and this is supported by the fact that neither of the organisms possesses the power of transforming *l*-aspartic acid into fumaric acid.

The relative rates of oxidation of succinate and fumarate vary very widely with different cells. The greatest variation is seen in the cases of muscle and brain tissue, with which the oxidation of fumarate is small compared with that of succinate. The reverse of this, when the oxidation of fumarate is seen to be even greater than that of succinate, occurs with *B. pyocyaneus*, an

observation entirely in harmony with the facts relating to the relative rates of fermentation of succinate and fumarate by this organism [Quastel, 1924].

The oxidations of fumarate and *l*-malate are very much more dependent on the age or condition of the organism than is the oxidation of succinate. Storing the organisms for a few days at 0° brings about a very marked decrease in the ability of an organism to oxidise fumarate or *l*-malate. It is this very high sensitivity of the oxidation to relatively slight changes in the cell which, doubtless, accounts for the fact that with muscle, even when used quite fresh, variable results are obtained for the oxidation of *l*-malate. To obtain reliable results with bacteria, these organisms must be used as soon as possible after their preparation. With *B. coli*, freshly prepared, the rate of oxidation of succinate is usually roughly twice that of fumarate; on storing the organism for a few days at 0° the rate of oxidation of succinate becomes as much as ten times that of fumarate—a ratio approximating to that found in muscle or brain tissue.

The effect on succinate oxidations of the addition of fumarate, malate or aspartate.

It is now well known both from studies of bacteria and of muscle that the enzyme accomplishing the activation of succinate as a hydrogen donator will bring about the activation of fumarate as a hydrogen acceptor. It follows that fumarate is adsorbed at the succinate-activating enzyme and hence that it should compete with succinate for this enzyme. The addition, therefore, of fumarate to succinate (both at "saturation" concentrations) should bring about a diminution in the velocity of oxidation of the latter, the amount of diminution being dependent upon the relative degrees of adsorption. Experiment shows this to be the case. Usually it is found (see Table I) that the oxygen uptake of a mixture of succinate and fumarate is the average of that due to succinate alone and that due to fumarate alone, this being the case whether the oxidation of fumarate is small or large compared with that due to succinate. An exception to this rule has been found with muscle and the interesting question arises as to whether the adsorption of fumarate at the succinate enzyme is the same whatever the source of the enzyme. There is not yet, however, sufficient experimental evidence available to decide this point.

Incidentally the fact that fumarate inhibits succinate oxidation is in harmony with the hypothesis that the main course of oxidation of succinate is through fumarate. Were there a different means of oxidising succinate, it would have been anticipated that the addition of fumarate would have increased the rate of oxidation found in presence of succinate alone.

Since fumarase is present in the organisms and tissues under investigation, the effect of adding *l*-malate is virtually the same as adding an equivalent concentration of fumarate, for both give rise to the same equilibrium mixture. Experiment shows that the inhibiting action of *l*-malate is rather less than that

of fumarate, an effect presumably to be accounted for by the time factor involved in the equilibrium concentration of fumarate being formed from the malate. For instance *M. lysodeikticus* is slow compared with *B. coli* in transforming fumarate into malate, and accordingly we find that the degree of inhibition of succinate oxidation by malate is less with the former organism than with the latter.

Aspartate, in the presence of those organisms which can convert aspartic acid into fumaric acid, inhibits the oxidation of succinate. With those organisms which do not possess this power, however, aspartate increases the rate of oxidation found with succinate. Thus with *B. coli*, *B. acidi lactici* and *B. proteus*, which are organisms capable of forming *l*-aspartic acid from fumaric acid, the effect of aspartate is to inhibit succinate oxidation. Now, were the aspartate directly oxidised, instead of passing through fumarate and malate, the action of aspartate would be to increase the rate of oxidation in presence of succinate. With the strict aerobes *B. alkaligenes*, *B. subtilis* and *M. lysodeikticus* this actually occurs, there being a marked increase in the rate of oxidation on the addition of aspartate to succinate. This shows clearly the difference in mode of attack on the amino-acid between the strict aerobes and the facultative anaerobes.

B. prodigiosus seems to behave, to a small extent, in this respect like a strict aerobe.

The interesting fact emerges, however, with the strict aerobes, that the rate of oxidation of a mixture of aspartate and succinate is often greater than the sum of the individual rates. This does not always occur and the phenomenon depends very largely on the condition of the organism.

The same phenomenon applies to muscle tissue. Here again the addition of aspartate increases the rate of oxidation due to succinate, the new rate sometimes being greater than the sum of the individual rates. The effect is reminiscent of Needham's observation [1930] that a mixture of glutamic and aspartic acids gives rise to more succinic and malic acids than the sum of the acids produced from glutamic and aspartic acids individually.

With brain tissue, *l*-aspartate appears to have but little effect on succinate oxidation.

Both with muscle and brain tissue fumarate and malate inhibit succinate oxidations, the effects being rather less than with bacteria.

It is quite clear, as far as aspartate oxidation is concerned, that, both with muscle tissue and the strict aerobes, the line of oxidation is not mainly through fumarate and malate. With the facultative anaerobes, on the other hand, this course would appear to be the predominant one. The alternative would be that for these organisms alone the aspartate molecule *per se* is highly adsorbed at the succinate enzyme—a hypothesis for which there is as yet no evidence.

The action of malonate.

It would be anticipated that if fumarate underwent a direct oxidation, this would take place at the enzyme which is known to activate the fumarate molecule, *i.e.* at the succinate dehydrogenase. Though the electrical theory of activation does not favour the possibility of the activated fumarate molecule taking up negative electricity (*i.e.* of becoming directly oxidised), there is no definite evidence so far that this does not in fact occur. Now it has been shown that malonate competes with succinate for the enzyme capable of activating the latter, and Cook [1930] has recently shown that this inhibitory action of malonate on succinate oxidation applies aerobically, as well as anaerobically, to *B. coli*. There is little doubt that the competing action of malonate with succinate for the latter dehydrogenase is a general phenomenon—applying to muscle and brain tissue (see Table I) as well as to various classes of bacteria.

It would be expected, therefore, that if fumarate is oxidised at the succinate enzyme, where it is known to be adsorbed, the presence of malonate would inhibit its oxidation by competition for the enzyme.

The action of malonate on fumarate oxidation provides, therefore, a good test of the possibility that fumarate undergoes an oxidation at the succinate enzyme.

Experiment shows that the action of malonate both on fumarate or malate oxidation is either nil or very small.

For instance with *B. coli*

Fumarate ($M/37$)	took up	423 mm.^{3} O_2	in 2 hrs.
Fumarate ($M/37$) + malonate ($M/15$)	,,	430	,,
l-Malate ($M/37$)	,,	427	,,
l-Malate ($M/37$) + malonate ($M/15$)	,,	400	,,

The fact that malonate has either little or no effect on fumarate and malate oxidations explains the wide variations observed in the degrees of inhibition by malonate on succinate oxidation with various organisms and tissues. In the case of those cells with which the rate of fumarate oxidation approaches that of succinate, malonate has a relatively small effect; for, in spite of the large effect of malonate on the succinate-fumarate reaction, fumarate is still produced in sufficient quantity to allow a relatively rapid uptake of oxygen. (It is worthy of note that the succinate-fumarate reaction requires only one atom of oxygen, whilst the complete oxidation of fumarate requires six atoms.) On the other hand, in the case of those organisms with which the fumarate oxidation is slow compared with that of succinate, malonate has a large effect, the action of the malonate on the succinate-fumarate reaction being predominant. This is the case with muscle and brain tissue with which the fumarate oxidation is normally small, or with organisms which have been stored so long or treated in such a way that the fumarate and malate oxidations have disappeared. Here the malonate inhibition ap-

proaches 90 % or an even larger value. With organisms, however, such as *M. lysodeikticus* or *B. pyocyaneus* where fumarate oxidation is relatively high the malonate inhibition may approach only 30 %.

The small or negligible action of malonate on fumarate oxidation is, definitely, evidence against the view that fumarate might undergo a direct oxidation at the succinate enzyme.

The action of oxalate.

If the oxidation of fumarate proceeds largely through *l*-malate it follows that an agent which inhibits malate oxidation will also inhibit fumarate oxidation. Such an agent is oxalate. The following results were obtained with *B. pyocyaneus*, all the substrates being initially present at a concentration of *M*/15 and the oxalate as the potassium salt.

l-Malate	took up	551	mm.^{3} O_2 in 90 mins.
l-Malate + oxalate	,,	330	,,
Fumarate	,,	600	,,
Fumarate + oxalate	,,	315	,,
Succinate	,,	547	,,
Succinate + oxalate	,,	409	,,

It will be seen that oxalate exercises an inhibitory action on both malate and fumarate oxidations—the inhibition being greater than on that of succinate. Oxalate has not a large inhibitory action in the succinate-fumarate reaction [Quastel and Wooldridge, 1928] but it is to be expected it will appreciably inhibit the total oxidation of succinate, since the latter passes through fumarate and malate.

The fact that the inhibitions of fumarate and *l*-malate oxidations by oxalate are of the same order of magnitude is clearly evidence in favour of the view that fumarate oxidation proceeds *via l*-malate.

It is worthy of note that toluene, which eliminates the oxidation of fumarate by *B. coli* [Cook, 1930], also eliminates the oxidation of *l*-malate by this organism.

Oxidation of succinate to *l*-malate.

Summing up the evidence so far it appears:

(1) the velocities of oxidation of fumarate and *l*-malate are usually of the same order of magnitude;

(2) malonate, which inhibits the succinate-fumarate reaction, has little action on the oxidation of either fumarate or *l*-malate;

(3) oxalate inhibits the oxidation of fumarate and *l*-malate to about the same extent, the inhibition being greater than that observed with succinate.

Though this evidence, on the whole, is in favour of the view that the oxidation of fumarate proceeds largely through *l*-malate there is as yet no direct evidence that succinate does not suffer another oxidation (*e.g.* direct to *dl*-malate) besides that of proceeding, in the first place, to fumarate.

This evidence may be secured from a quantitative study of the oxidation of succinate in presence of those tissues or organisms with which the oxidation of fumarate or malate is small or nil. In presence of such cells, one atom of oxygen should be taken up per molecule of succinate forming an equilibrium mixture of fumarate and *l*-malate in the ratio of 1:3. Thus the uptake of 1 g.-atom of oxygen should give rise to $\frac{3}{4}$ g.-mol. of *l*-malate. If succinate suffered an oxidation other than that through fumarate, the uptake of 1 g.-atom of oxygen would lead to less than $\frac{3}{4}$ g.-mol. *l*-malate. If, for instance, it proceeded first to *dl*-malate, and this to a mixture of *d*-malate, *l*-malate and fumarate the final rotation observed would not only be smaller than if fumarate were the first step but of the opposite sign.

For experiment, succinate at an initial concentration of $M/15$ was oxidised in presence of (1) muscle tissue, with which the fumarate oxidation was very small compared with that of succinate; (2) a suspension of *B. coli* which had been treated with toluene so as to eliminate the oxidation of fumarate.

The oxidation was carried out in the Barcroft apparatus and after a certain period the oxygen uptake was read, and the solution in the Barcroft cup made up to 5 cc. with water. 1 cc. glacial acetic acid was added and the whole mixed with 10 cc. 14·2 % ammonium molybdate solution. After filtering or centrifuging, the clear solution was examined polarimetrically. Using the mercury green line a rotation of 1° (2 dm. tube) was equivalent to 9·65 mg. *l*-malic acid. The experimental results were as follows.

(1) *With muscle tissue:*

Oxygen uptake at 37° and 760 mm.	357 mm.^{3}
Rotation observed	0·30°
Rotation calculated on the basis of succinate being oxidised entirely through fumarate...	0·30°

(2) *With* B. coli *treated with toluene:*

Oxygen uptake at 37° and 760 mm.	1222 mm.^{3}
Rotation observed	1·06°
Rotation calculated	1·02°

These results clearly indicate that if succinate normally undergoes some other oxidation, besides that of proceeding through fumarate, the amount of this oxidation must be exceedingly small.

The entire evidence is now in support of the view that the normal course of biological oxidation of succinate lies largely, if not entirely, through fumarate and *l*-malate.

Warburg's respiratory enzyme.

The inhibition of succinate oxidation by malonate in presence of the intact cell bears upon the views held by Warburg of the "respiration enzyme" of the cell. These views are best expressed in his own words [Warburg, 1930] "...if different oxidases occur in an extract of one kind of cell they are not

enzymes which were preformed in the living cell, but transformation and decomposition products of a substance uniform in life. Uniformity of respiration enzyme and multitude of oxidases in the extracts do not constitute a contradiction."

Presumably, then, on this view the system responsible for the oxidation of succinate in the intact cell is identical with that which is responsible for the oxidation of fumarate, or lactate, or (say) *p*-phenylenediamine. This view leaves no room for the existence of specific dehydrogenases in the intact cell and supposes that the effects of treatment, or extraction, of a cell, on the various oxidations, are not to eliminate certain dehydrogenases but to affect a common oxidase in such a way that the less "sensitive" substances are no longer oxidised whilst the more sensitive are still attacked.

That this view cannot be true follows not only from the fact that with the bacteria the dehydrogenases are selectively poisoned [Quastel and Wooldridge, 1927], but from quantitative evidence with the intact cell. It was shown for instance that the oxidase systems for succinate and glucose differed from each other. Were there a common oxidase for these substances in the intact cell, then the rate of oxidation of a mixture of the substances at their saturation concentrations would not be greater than the higher of the individual rates. Experiment showed that the oxidation rate was the sum of the individual rates.

In this communication it has been shown that with *B. alkaligenes*, *B. subtilis* and *M. lysodeikticus* the velocity of oxidation of a mixture of succinate and aspartate is greater than either of the individual rates—a fact showing the presence of two distinct activating systems in the intact cell.

Attention, however, might be drawn, in this connection, to the inhibiting action of malonate. It has been shown previously by the methylene blue method that this substance acts reversibly on *B. coli* [Quastel and Wooldridge, 1928]. Moreover malonate does not injure the cell, for the organism is capable of prolific growth in presence of it.

Now it would be expected, if succinate and *p*-phenylenediamine are oxidised by the same system in the intact cell, that malonate which inhibits succinate oxidation would also inhibit the *p*-phenylenediamine oxidation. Experiment shows that this is not the case.

Thus, with brain tissue (0·5 g.):

p-phenylenediamine (21 mg.) took up in 2 hrs. 1206 mm.^{3} O_2.
p-phenylenediamine (21 mg.) + malonate (*M*/15) took up in 2 hrs. 1201 mm.^{3} O_2.
p-phenylenediamine (21 mg.) + oxalate (*M*/15) took up in 2 hrs. 1132 mm.^{3} O_2.

Neither malonate nor oxalate appreciably inhibits *p*-phenylenediamine oxidation under conditions such that succinate oxidation is markedly inhibited by malonate.

The conclusion is clear that, for a consistent interpretation of the results of biological oxidations, it is necessary to picture the existence in the intact cell of distinct active centres, or dehydrogenase systems, each concerned with the activation of a certain type of hydrogen donator. Possibly, as already suggested [Quastel and Wooldridge, 1927], the process of treating or extracting a cell alters the activating range of each centre, but that more than one distinct activating centre exists in the intact cell there can be no question. The oxidase which is concerned with the activation of molecular oxygen is quite distinct from the systems already referred to. Whether there is one such oxidase or a number in the intact cell is still a problem for further investigation.

SUMMARY.

1. It has been shown from quantitative evidence that the normal course of biological oxidation of succinate proceeds largely, if not entirely, through fumarate and *l*-malate. The evidence results from

(*a*) a comparative study of the oxidations of fumarate and *l*-malate in presence of various organisms and tissues;

(*b*) a comparison of the action of malonate and of oxalate on the oxidation of fumarate and *l*-malate;

(*c*) measurements of *l*-malic acid formed from succinic acid; the calculated quantities are in close agreement with those observed.

2. Fumarate and *l*-malate inhibit the oxidation of succinate in presence of various bacteria and of muscle and brain tissues.

3. It is shown that the strict aerobes, *B. alkaligenes*, *B. subtilis* and *M. lysodeikticus*, and also muscle tissue, attack *l*-aspartate in a different manner from the facultative anaerobes, *B. coli*, *B. proteus* and *B. acidi lactici*. The latter oxidise the acid through fumaric and *l*-malic acids.

4. Malonate not only inhibits the oxidation of succinate by bacteria but also that by muscle and brain tissues, the degree of inhibition being greatest with these tissues. It is shown that the wide variation with different cells in the degrees of inhibition by malonate is associated with differences between velocities of oxidation of fumarate and those of succinate.

5. Neither malonate nor oxalate inhibits the velocity of oxidation of *p*-phenylenediamine by brain tissue.

6. These results are discussed in relation to Warburg's views on the "respiration enzyme" of the cell.

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XVII. A STUDY OF THE MECHANISM OF THE DEGRADATION OF CITRIC ACID BY *B. PYOCYANEUS* (*PSEUDOMONAS PYOCYANEA*).

II. ACTION OF *B. PYOCYANEUS* ON SUCCINIC ACID.

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In Part I [Butterworth and Walker, 1929] it was shown that the products of the action of *B. pyocyaneus* on ammonium citrate include malonic acid and a small quantity of succinic acid, and it was suggested that the latter arises by simultaneous decarboxylation and dehydrogenation of two molecules of the malonic acid in presence of a hydrogen acceptor. Pyruvic acid, which might well be derived from the acetone also produced in the ammonium citrate cultures, could exercise such a function, being thereby converted to lactic acid or, under certain conditions, to propionic acid. Support for this hypothesis is to be found in the experiments of Aubel and Cambier [1922], who detected lactic acid in cultures of *B. pyocyaneus* on solutions of the salts of both citric and pyruvic acids. Further, as will be evident from results described in the course of the present communication, the fact that propionic acid has been shown to be an end-product of the action of *B. pyocyaneus* on ammonium succinate [Aubel, 1921; Quastel, 1924] supports the same view. We have also obtained additional experimental evidence in favour of such a possibility by establishing the fact that addition of small quantities of acetone to cultures of *B. pyocyaneus* on calcium acetate media is followed by the formation therein of lactic acid.

In view of the occurrence of succinic acid in the citrate cultures, and of the fact that propionic acid is formed by the growth of *B. pyocyaneus* on succinate media, it was considered of interest to make a closer study of the behaviour of succinic acid under the attack of this organism. It was considered unlikely that the propionic acid originates in the simple decarboxylation of succinic acid under the influence of the bacillus, as suggested by Aubel, since such a view is not in harmony with the known facts of organic chemistry, and its formation was regarded as more probably due to the following series of changes:

$$COOH.CH_2.CH_2.COOH \rightarrow COOH.CH_2.CH(OH).COOH \rightarrow COOH.CH_2.CO.COOH \rightarrow CH_3.CO.COOH \rightarrow CH_3.CH(OH).COOH \rightarrow CH_3.CH_2.COOH.$$

In the comprehensive work of Van Niel [1928] on the chemistry of the propionic acid bacteria, the formation of propionic acid from both pyruvic acid and lactic acid was verified beyond doubt.

Adopting the above tentative scheme as a basis of enquiry, search for the presence of malic acid was made in cultures of *B. pyocyaneus* on ammonium succinate, and its formation was demonstrated in several experiments after fermentation had been in progress for about 9 days. The acid was detected by Denigès' mercuric acetate reagent [1900] and in subsequent experimental fermentations it was isolated and examined in detail, and, in every instance, it was found to consist of a mixture of the *dl*- and *l*-forms. Since *B. pyocyaneus* does not effect racemisation of the active forms of malic acid, and since we have proved by control experiments that *l*-malic acid is unaltered by the treatment adopted for its isolation from the cultures, there remain only two possible sources from which the *dl*-malic acid could have been derived, namely, (*a*) from the succinic acid by the action of activated hydrogen peroxide, for *B. pyocyaneus* is known to contain a peroxidase system, and (*b*) from oxaloacetic acid by symmetrical reduction. We regard (*a*) as the more probable alternative, for biological reduction has usually been observed to yield asymmetric compounds when structure has permitted optical activity and, further, the fact that *B. pyocyaneus* is rich in catalase is not an argument against (*a*), since it has been demonstrated clearly [Chodat and Pasmanik, 1907; Thurlow, 1925] that a partition of hydrogen peroxide between catalase and peroxidase occurs when both enzymes are present.

In order to throw light on the source of the *l*-acid the bacillus was then cultivated on media in which *dl*-malic acid constituted the sole source of carbon and, in the several experiments undertaken, such media invariably developed dextrorotatory properties. They also gave positive reactions to the test for pyruvic acid devised by Quastel [1924] and were free from lactic acid. Hence it follows that the *l*-malic acid obtained by the fermentation of ammonium succinate is not derived through resolution by the bacillus of preformed *dl*-malic acid, but must have its genesis in the asymmetric addition of water to fumaric acid arising from the dehydrogenation of succinic acid. In this connection it is to be noted that Stent, Subramaniam and Walker [1929] have recently shown that succinic acid yields a mixture of *dl*- and *l*-malic acids when submitted to the attack of *Aspergillus niger*, and that the latter organism renders solutions of *dl*-malic acid dextrorotatory, whilst Challenger and Klein [1929] have established the fact that the same strain of this mould produces high yields of *l*-malic acid from potassium fumarate, whence it would appear that the degradation of succinic acid is achieved in precisely the same manner by both *B. pyocyaneus* and *A. niger*.

Quastel [1924], Quastel and Whetham [1924] and Quastel, Stephenson and Whetham [1925], in the course of studies of the reactions induced by resting bacteria have considered the cases of the equilibria which are set up (*a*) between fumaric acid and *l*-malic acid in the presence of resting *B. coli*, and (*b*) between

succinic acid and fumaric acid in the presence of methylene blue and resting *B. pyocyaneus*. Their experimental results are later interpreted as special cases of the general theory of the mechanism of oxidation and reduction *in vivo*, postulated by Quastel [1926], in which the breakdown of succinic acid by resting bacteria is assumed to proceed by initial formation of an activated form of fumaric acid, symbolised as $COOH—CH_2—\check{C}—COOH$, and regarded as identical with the activated form into which it is assumed fumaric acid is first converted under similar enzymic influence. This explanation is an elaboration and to some extent a modification of Wieland's dehydrogenation hypothesis, and if it also applied to the behaviour of succinic acid when submitted to the attack of actively growing *B. pyocyaneus* under aerobic conditions, we should expect to obtain products identical with those which arise by the action of this organism on fumaric acid. Such, however, is but partially the case for, as we have just seen, the breakdown of the succinic acid in this instance is largely achieved by what appears to be a peroxidase reaction (leading to the formation of racemic malic acid), which proceeds simultaneously with the dehydrase and fumarase reactions affording respectively fumaric acid and thence *l*-malic acid.

According to Quastel [1924] the growth of *B. pyocyaneus* on media containing salts of fumaric acid is very vigorous and malic acid cannot be detected among the products, of which the first to be identified is pyruvic acid. Further, Quastel and Whetham [1924] found malic acid to be incapable of donating hydrogen to methylene blue in the presence of the resting organism. As a result of these observations they concluded that the conversion of fumaric acid to pyruvic acid probably proceeds through the intermediate formation solely of oxaloacetic acid

$$COOH—CH = CH—COOH \rightarrow COOH—CH_2—CO—COOH \rightarrow CH_3—CO—COOH + CO_2.$$

In a later paper, however, Quastel [1926] brings forward evidence which vitiates this conclusion, for he shows that the effect of an activating field on malic acid is to produce a certain amount of activated fumaric acid which functions as a hydrogen acceptor

$$COOH—CH(OH)—CH_2—COOH \rightleftarrows COOH—\check{C}—CH_2—COOH + H.OH,$$

in consequence of which he states that malic acid, after activation, behaves both as a hydrogen acceptor and donator, and considers that probably the donating properties are obscured in the experiments with *B. coli* by the accepting powers.

In our view the formation of *l*-malic acid in cultures of *B. pyocyaneus* on succinate media which, as we have demonstrated, can only have taken place by asymmetric addition of water to fumaric acid arising by preliminary dehydrogenation of the succinic acid, renders it more probable that the pyruvic acid produced in cultures of *B. pyocyaneus* on fumarates arises through the changes:

$$COOH.CH : CH.COOH \rightarrow COOH.CH_2.CH(OH).COOH \rightarrow COOH.CH_2.CO.COOH \rightarrow CH_3.CO.COOH + CO_2,$$

than by the direct oxidation of fumaric acid to oxaloacetic acid. This opinion must not be construed as belief on our part that direct oxidation of this type cannot take place, for Quastel's activation theory [1926] indicates a reasonable mechanism by which such a change could, in certain cases, be effected.

Experimental.

The strain of *B. pyocyaneus* employed was that used in Part I of this investigation, namely, Fildes III, No. 1999, from the National Collection of Type Cultures, Lister Institute. The substances under investigation were submitted to its action in Ringer's inorganic salt solution, the various media being adjusted to p_H 7·4 prior to inoculation.

Enzymic conversion of acetone to lactic acid.

Forty flasks of 500 cc. capacity were each charged with 5 g. of calcium acetate (Kahlbaum) and 1·0 g. of ammonium nitrate in 250 cc. of Ringer's solution. After sterilisation, 0·5 g. of pure acetone was delivered from a sterile pipette into each flask, followed by an inoculum of 1 cc. of a 24 hours old culture of the organism on the same medium. Incubation was conducted for 3 days at 25°, after which the contents of the flasks were combined and concentrated somewhat by distillation. The distillate contained no acetone. The residue was evaporated to dryness on the steam-bath. Preliminary tests having indicated that this material contained calcium lactate, a portion (20 g.) was distilled with 100 cc. of sulphuric acid (50 % concentration) until 30 cc. of distillate had passed through the ice-cooled condenser. The acetic and formic acids in this distillate were neutralised with sodium hydroxide and the solution was then distilled through the ice-cooled condenser until 20 cc. of distillate were obtained. This gave a strongly positive test for acetaldehyde by Rimini's reaction [1904]. It was mixed with a solution of *p*-nitrophenylhydrazine (5 g.) in acetic acid (40 cc. of 50 % concentration) and the precipitate was washed, dried and recrystallised from alcohol; M.P. 128° alone or in admixture with an authentic specimen of acetaldehyde *p*-nitrophenylhydrazone (M.P. 128°). Yield, 0·8 g. (found, N, 23·8; calc. N, 23·46 %).

Another portion (22 g.) of the original dry residue presumed to contain calcium lactate was heated with 50 % sulphuric acid as before, and the distillate was neutralised with sodium hydroxide and evaporated to dryness. The residue was extracted several times with methyl alcohol in order to remove sodium acetate, and the residual small quantity of insoluble solid material was taken up in 10 cc. of water and treated with 2 g. of lead nitrate dissolved in 10 cc. of water, when a precipitate formed and was removed. It was washed with cold water and recrystallised from hot water, yielding 0·8 g. of pure lead formate (found, Pb, 69·4; calc. 69·7 %).

A control experiment in which pure calcium lactate was distilled with sulphuric acid gave acetaldehyde and formic acid as above. In a blank experi-

ment no trace of lactic acid was detected in a solution of pure calcium acetate fermented in the absence of acetone.

Detection of malic acid in cultures of B. pyocyaneus *on ammonium succinate.*

The most delicate test for the detection of malic acid is that of Denigès [1900], and it was found that this could be combined with the use of 2 : 4-dinitrophenylhydrazine whereby a means was provided for obtaining quickly a rough approximation of the concentration of malic acid in a solution containing only a trace. Thus, dilute aqueous solutions were made up containing respectively 0·1, 0·05, 0·033, 0·025 and 0·02 % of malic acid. 25 cc. of each solution were treated with 3 to 4 cc. of Denigès's mercuric acetate solution, then boiled and filtered. Slight excess of a 2 % potassium permanganate solution was added to each, drop by drop, whilst the solutions were maintained at the boiling point. The precipitates of the basic mercury salt of oxaloacetic acid which were thus obtained were allowed to settle in test-tubes, the clear supernatant liquid was decanted and the precipitates were then each treated with 5 cc. of a saturated solution of 2 : 4-dinitrophenylhydrazine hydrochloride in 2*N* hydrochloric acid. This gave rise to slight precipitates of yellow crystalline material in all cases in which the malic acid had been present originally at a concentration of 0·025 % or higher. These crystals consist probably of a mixture of the 2 : 4-dinitrophenylhydrazones of pyruvic acid and acetaldehyde, since treatment of the basic mercury salt of oxaloacetic acid with cold dilute hydrochloric acid yields a solution which smells strongly of acetaldehyde, and which gives a positive reaction for pyruvic acid when treated with an alcoholic solution of guaiacol and strong sulphuric acid (Quastel's test [1924]). The results of the above-described experiments may be tabulated thus:

% concentration of malic acid in solution	0·1	0·05	0·033	0·025	0·020
Volume (cc.) of 2 % $KMnO_4$ solution equivalent to 25 cc. of malic acid solution	0·56	0·30	0·20	0·15	0·10
Quantity of 2 % $KMnO_4$ solution added (drops)	12	9	7	5	4
Precipitate	+	+	+	+	+
Action of 2 : 4-dinitrophenylhydrazine	Yellow precipitate and golden opacity			Golden opacity. Precipitate on standing	Opalescence only

This information was then utilised in subsequent experiments in which it was sought to prove the presence of malic acid.

Ammonium succinate (30 g.) was dissolved in Ringer's solution (1500 cc.) and this mixture was distributed equally between 6 flasks of 500 cc. capacity. After sterilisation, inoculation was performed by addition to each flask of 1 cc. of an actively fermenting culture on the same medium, and incubation was conducted at 34°. At intervals of 7 hours portions of 25 cc. were withdrawn

and tested with 2 % $KMnO_4$ solution in the presence of mercuric acetate solution at the boiling point. After 3 days this test for malic acid was positive, whereupon 700 cc. of the culture solution were treated with these reagents and yielded 1·5 g. of the characteristic basic mercury salt of oxaloacetic acid which, on subsequent distillation with 5 g. of sodium iodide in 25 cc. of water, gave traces of acetaldehyde (Rimini's test). After 11 days the remaining contents of the culture flasks were combined and treated as above, when 18 g. of moist mercury salt were obtained. This was distilled with sodium iodide (30 g.) in water (150 cc.) and the well-chilled distillate was conducted beneath the surface of a solution of 2 : 4-dinitrophenylhydrazine hydrochloride (250 cc.) prepared according to the particulars given by Brady and Elsmie [1926]. The resultant precipitate (0·6 g.) was collected, washed, dried and recrystallised several times from hot ethyl alcohol, when it formed glistening orange-yellow crystals, M.P. 162°. This melting point was not depressed by admixture with a pure specimen of acetaldehyde 2 : 4-dinitrophenylhydrazone (found, by micro-method: N, 24·5; calc. N, 25·0 %).

Isolation of dl-*malic acid together with* l-*malic acid from cultures of* B. pyocyaneus *on ammonium succinate.*

Exp. 1. 2500 cc. of a 2 % solution of ammonium succinate in Ringer's solution, after adjustment to p_H 7·4, were equally distributed between 10 flasks of 500 cc. capacity. After sterilisation these were inoculated at the rate of 1 cc. per flask with an active culture which had grown for 24 hours on a medium of the same composition. Incubation was carried out at 34° and the flasks were carefully shaken twice daily in order to aerate the media. A positive test for malic acid was obtained on the fifth day, and increased in intensity up to the ninth day, whereupon the contents of all the flasks were combined, acidified with a little glacial acetic acid, and digested on the water-bath to coagulate the culture. The solution was filtered, further concentrated to 700 cc., treated with 140 g. of crystalline lead acetate in 200 cc. of water, and then made slightly alkaline with ammonia, after which 500 cc. of 95 % alcohol were added. Two days later the precipitate of lead salts was removed at the pump, washed with 30 % alcohol and dried. The material was triturated in a mortar and suspended in 100 cc. of water, and the lead removed by H_2S. On subsequent concentration to 75 cc. crystals of succinic acid separated on standing. These were removed and the mother liquor was then concentrated to 10 cc., when a further crop of succinic acid separated and was also removed. The residual syrupy liquid was neutralised with sodium hydroxide, diluted with water to 56 cc. and 4 cc. of glacial acetic acid were added. 25 cc. of this solution were mixed with 10 cc. of an 8 % solution of uranium acetate, and after standing in the dark showed a rotation of − 2·4° (Ventzke scale). 25 cc. of a 1 % solution of authentic sodium *l*-malate plus 10 cc. of an 8 % uranium acetate solution showed, on similar treatment, a rotation of − 13·7°. Assuming

the optical activity of the fermentation product to be due to *l*-malic acid, this result represents a yield of approximately 0·1 g. of the latter.

Exp. 2. This was a repetition of Exp. 1, with the exception that the fermentation was allowed to continue for a longer time. Malic acid was first detected on the 4th day. On the 14th day the bacteria were still active and a sample of the liquid gave a positive test for the presence of pyruvic acid when tested by Quastel's method. The whole of the culture medium was then worked up as in the previous experiment and the acid liquid was concentrated until succinic acid separated. The latter was removed and the process of concentration and removal of succinic acid was repeated until finally a viscous syrup, nearly free from succinic acid, was obtained. This was ground with anhydrous sodium sulphate and extracted with dry ether. The ethereal solution was concentrated to 20 cc. and treated with light petroleum until a faint turbidity appeared. On slow evaporation a brownish crystalline product was obtained. This sintered at 96° and a portion of it finally melted at 165°, hence it evidently still contained traces of succinic acid. The material was then shaken with 5 cc. of water and 10 cc. of ether. The aqueous extract, now entirely free from succinic acid, was almost neutralised with sodium hydroxide, evaporated to 5 cc. and heated on the water-bath with 0·8 g. of *p*-nitrobenzyl bromide in 10 cc. of alcohol. The product was recrystallised twice, when it had M.P. 114°. This value lies between that of the di-*p*-nitrobenzyl ester of *l*-malic acid (124–125°) and that of the di-*p*-nitrobenzyl ester of the *dl*-acid (109°). A mixture of the *dl*- and *l*-forms of malic acid in the ratio of 55 parts of the former to 45 parts of the latter was made from authentic materials and treated with *p*-nitrobenzyl bromide as above. The product after two recrystallisations had M.P. 115° and appeared identical with the esterified culture product.

Exp. 3. 3·5 litres of 2 % ammonium succinate medium were inoculated as before, but the fermentation was conducted at a lower temperature (30°). On the 13th day the malic acid test became strongly positive, whereupon the whole of the culture solution was concentrated to a syrup. Phosphoric acid was added and the mixture was treated with ether for several hours in a continuous-extraction apparatus. This afforded a light brown solid which was then treated with charcoal in hot aqueous solution. On evaporation of the filtered liquid there remained 27 g. of colourless crystals, M.P. 164°. These were redissolved in hot water and fractionally crystallised, when 7 g., M.P. 139–140°, were obtained. This was redissolved in water and extracted with ether. The aqueous layer on evaporation yielded 2·5 g. of slightly deliquescent crystals, M.P. 114–116°, and the ether extract gave material which, on further fractional crystallisation from hot water, afforded a small quantity of pure *dl*-malic acid, M.P. 130°. The substance of M.P. 114–116° gave tests for pure malic acid (found: equivalent, 66·4; calc. 67·0; found by micro-method: C, 35·2, 35·3; H, 4·4, 4·5; calc. C, 35·8; H, 4·5 %). A portion (0·111 g.) on polarisation in the presence of uranium acetate by Dakin's method [1924] gave a reading of − 6·3° (Ventzke scale) which indicated that 40 % of the material was *l*-malic acid. This was

confirmed by reference to a fusion-point curve of mixtures of *dl*- and *l*-malic acids which was constructed from determinations of the melting points of known mixtures. From this curve it was seen that a mixture of 60 % *dl*-acid with 40 % *l*-acid melts at 117°. A further small portion (0·1 g.) of the material of M.P. 114–116° was polarised in the presence of 10 % ammonium molybdate solution (Williamson's method [1918]), when a reading of + 2·1° (Ventzke scale) was observed, a final confirmation of the presence of *l*-malic acid. The remainder of the material was then fractionally crystallised from ether-light petroleum, when a very small amount of pure *dl*-malic acid, M.P. and mixed M.P. 130°, was finally separated.

Development of dextrorotatory activity in cultures of B. pyocyaneus *on* dl-*malic acid.*

Pure *dl*-malic acid (60 g.) was dissolved in 2 litres of Ringer's solution and neutralised by addition of approximately 50 cc. of concentrated ammonia solution (sp. gr. 0·88). The solution was divided between eight flasks, sterilised, and adjusted to p_H 7·4 by addition of hydrochloric acid. After inoculation, followed by incubation at 34° for 7 days, the cultures were combined and evaporated to dryness. The residue was redissolved in a little water, acidified with phosphoric acid and extracted with ether as in Exp. 3 for several hours. The ether extract was dried and evaporated to a syrup which slowly crystallised in a desiccator. The material was tested for lactic acid by distillation with sulphuric acid, but the distillate showed no reaction for acetaldehyde, hence lactic acid was absent. Two quantities (0·578 g. and 0·711 g.) of the crystals were dissolved in 80 cc. of distilled water in each case. The solutions were clarified with charcoal, neutralised with potassium hydroxide, treated with a drop of glacial acetic acid and then each made up to 100 cc. A portion (10 cc.) of one of the solutions showed a rotation of + 5·4° (Ventzke scale) in the presence of uranyl acetate; a further 10 cc. showed a rotation of − 7·8° (Ventzke scale) in presence of ammonium molybdate. Hence Williamson's test [1918] for the presence of *d*-malic acid was positive. The specific rotations of the two solutions were then determined by Dakin's method, when an average value of 301·5° was found, corresponding to the presence of 62·5 % of *d*-malic acid in the crystals.

The whole experiment was repeated, the fermentation being allowed in this case to continue for 14 days.

Polarimetric examination of the product demonstrated the presence of 70 % of *d*-malic acid.

During the fermentations of *dl*-malic acid described above the presence of pyruvic acid was detected in the media by Quastel's test, the reaction being positive after 21 hours' incubation, and intense colorations were obtained after 45 hours. The concentration of this acid was too low, however, to permit of its isolation, only traces of precipitate being obtained with ketonic group reagents.

SUMMARY.

1. The results described in the present paper, when considered in conjunction with those recorded by Butterworth and Walker [1929], show that the growth of *B. pyocyaneus* on salts of citric acid results in precisely the same reactions as occur during the breakdown of this acid by the mould *A. niger* [*cf.* Challenger, Subramaniam and Walker, 1927].

2. The action of *B. pyocyaneus* on succinates also leads to the formation of the same products, *dl*-malic acid and *l*-malic acid, as are obtained by the action of *A. niger* on succinates. The evidence indicates that the *dl*-malic acid arises by direct hydroxylation of succinic acid and the *l*-malic acid by dehydrogenation of succinic acid to fumaric acid, followed by asymmetric addition of water to the latter.

3. From these observations in conjunction with other experimental data it is rendered likely that the formation of pyruvic acid from fumaric acid under the influence of *B. pyocyaneus*, as studied by Quastel, proceeds by the stages fumaric acid → *l*-malic acid → oxaloacetic acid → pyruvic acid, and not in the more direct manner which experiments with methylene blue would appear to indicate.

4. A method, based on Denigès' mercuric acetate test, is described for the detection and approximate estimation of traces of malic acid in solution.

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XVIII. THE ANTISCORBUTIC POTENCY OF APPLES. II.

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Some recent publications [Bracewell, Hoyle and Zilva, 1930, 1, 2] have dealt with an exploratory investigation instituted with the object of ascertaining whether a functional relationship exists between the antiscorbutic activity and the physiological condition of the apple fruit. With this end in view the potency of a number of English and imported varieties of known environment was assessed and the influence of empirical methods of storage and of heat on the vitamin content of apples was studied. The following salient facts emerged.

(1) The Bramley's Seedling is markedly more potent than the other varieties tested, which definitely vary in activity among themselves but to a smaller extent.

(2) There is no indication, so far, that "age of tree," soil or season has any bearing on the antiscorbutic activity of the apple.

(3) There is little deterioration of the vitamin when apples are stored either at 1° in the air (cold store) or at 10° in an atmosphere of approximately 10 % oxygen, 10 % carbon dioxide and 80 % nitrogen ("gas" store); the loss is, however, greater in the case of the "gas" stored apples.

(4) Heating Bramley's Seedling apples in their skins at about 115° for approximately 50 minutes has little effect upon their antiscorbutic activity.

While these results offer no indication so far whether vitamin C has any physiological function in the plant, they nevertheless point to the fact that the following up of this enquiry is advisable, not only from the vitamin aspect but also from the botanical point of view. Without drawing any broad lines of demarcation, the investigation is now being continued in collaboration with groups of workers interested in different fields of pomological research. In view of the lengthy nature of the investigation and of the seasonal character of the experimental material, it is proposed, for the sake of convenience, to record the results at suitable intervals even if they do not yield information from which definite conclusions can be drawn at the time.

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The present communication deals with:

(1) The antiscorbutic activity of further apple varieties.

(2) The effect of low temperatures and of maturity on the antiscorbutic activity of the apple.

(3) The effect of temperature of storage on the antiscorbutic activity of ageing apples.

(4) The distribution of the vitamin in various parts of the apple.

The technique utilised in these tests was precisely the same as that already described [Bracewell, Hoyle and Zilva, 1930, 2].

The antiscorbutic activity of further apple varieties.

The varieties tested were Newton Wonder and Lane's Prince Albert.

The Newton Wonder apples were taken from fifteen year-old trees grown on a flinty loam over chalk, near Eynsford, Kent.

The Lane's Prince Albert apples came off 15–20 year-old trees grown on a chalk soil at Exning, near Newmarket.

The apples after being picked were stored at a constant temperatue of 1° at the Low Temperature Research Station, Cambridge, and weekly consignments were delivered to London for testing purposes.

The test of the Newton Wonder apples began on 15th October, 1929, and that of the Lane's Prince Albert on 24th October, 1929.

Table I.

Dose g. daily	Lane's Prince Albert 1929	Newton Wonder 1929	Normal Bramley's Seedling 1929 Stored 3° 1st batch	King Edward 1929 Bristol High nitrogen	Worcester Pearmain 1928 Burwell	Normal Cox's Orange Pippin 1928 Burwell	Woodbine 1928 Bristol	Dabinett 1928 Bristol
3	±	±	+ + + ±	No protection	No protection	±	No protection	No protection
5	+ + +	+ ±	+ + + ±	±	No protection	+	?	+ + ±
10	+ + + +	+ + + +	+ + + +	+ + ±	±	+ + ±	+	+ + + +
20	+ + + +	Vitiated test	Test not finished, but fully protected for 60 days	+ + + ±	+ + +	+ + + ±	+ + + +	+ + + +

It will be seen from Table I, in which other varieties are incorporated for comparison, that the Newton Wonder showed an activity of the order of Cox's Orange Pippin whilst the Lane's Prince Albert, like the Dabinett, falls between the Bramley's Seedling and the Cox's Orange Pippin in vitamin activity. It will be of interest to ascertain whether further tests will confirm this result.

The effect of low temperature (− 20°) and of maturity on the antiscorbutic activity of the Bramley's Seedling.

In the first investigation of the series it was found that the early gathering of Bramley's Seedling made no appreciable difference to its vitamin C content. It was next of interest to ascertain whether in the still earlier stages of the life cycle of the apple the concentration of the vitamin is of the same order as when the fruit is ready for gathering. If that were so, it would indicate that the vitamin is either synthesised by the apple or that it is supplied to it as it grows on the tree. Alternatively, it might have been supposed that even at this early stage the fruit contains the same amount of vitamin as when it is fully grown. In this event the antiscorbutic potency of the immature apple tissue would have been proportionately higher than that of the mature apple.

Bramley's Seedlings, about 3·2 cm. in diameter and 23 g. in weight, gathered at the end of July, and fully grown apples of similar origin gathered in the middle of October were tested for comparison. The fruit was picked from 20 year-old trees grown on flinty loam over chalk near Eynsford, Kent.

The mature and immature apples were placed immediately after gathering in a constant-temperature room kept at − 20° at the Low Temperature Research Station at Cambridge, and weekly supplies were removed from the room and despatched to London in a well-insulated box. On arrival the fruit was transferred immediately to a refrigerator in which a temperature of approximately − 12° was maintained. Quantities necessary for the test were removed daily, thawed and brought up to a temperature suitable for feeding to the experimental animals.

The test on the immature Bramley's Seedlings began on 8th August, 1929, that on the normal[1] apples on 21st October, 1929 (1st batch). The normal fruit was also kept in the hard frozen condition until 20th February, 1930 (2nd batch), when it was again tested for its antiscorbutic potency.

Table II.

Dose g.	Frozen immature Bramley's Seedling 1929	Frozen normal Bramley's Seedling 1929, 1st batch	Frozen normal Bramley's Seedling 1929, 2nd batch	Normal Bramley's Seedling 1929 Stored 3° 1st batch
½	No protection	Not tested	Not tested	Not tested
1	±	,,	,,	,,
3	+ + +	+ +	+ +	+ + + ±
5	+ + + +	+ + + +	+ + + +	+ + + ±
7	+ + + +	Not tested	Not tested	Not tested
10	+ + + +	+ + + ±	+ + + +	+ + + +
20	Not tested	+ + + ±	+ + + +	Test not finished, but fully protected for 60 days

The results are summarised in Table II. When compared with similar apples kept at 3° the frozen apples show a slightly but definitely lower vitamin content. There are indications, however, that this small loss was not incurred

[1] The term "normal" is used throughout this paper to denote that the fruit was gathered at the normal time.

in the process of freezing but during the period which elapsed between the thawing of the fruit and its consumption by the experimental animals. Precisely the same titre was obtained with these apples after 4 month's storage.

The immature Bramley's Seedlings seem slightly more active than the normal apples. Had they, however, possessed the same total amount of vitamin as the fully grown apples, a daily dose of 0·5 g., or even less, of tissue would have been sufficient to protect a guinea-pig from scurvy. The amount of vitamin in the apple therefore increases as it grows on the tree.

The effect of temperature of storage on the antiscorbutic activity of ageing apples.

In view of the observations made that the loss in the antiscorbutic activity of apples is somewhat greater when the fruit is stored in an atmosphere of nitrogen, carbon dioxide and oxygen at 10° (*i.e.* "gas" storage) than when it ages in the air at 1°, it was thought desirable to ascertain whether this higher loss was brought about by the difference in the temperature or in the composition of the atmosphere of the two methods of storage. Bramley's Seedling apples gathered at the normal time, of the same origin as those which were employed in the preceding experiments, were therefore allowed to age in the air at 3° and at 10°. Unfortunately circumstances did not permit a comparison to be made with apples "gas"-stored at 10°. In the case of the Bramley's Seedling variety a displacement of the metabolic balance of the tissues occurs during storage at 1°, eventually leading to low-temperature breakdown, whereas at 3°, the metabolic activities of the fruit proceed normally; hence the choice of the latter temperature in this experiment.

The tests on the first batch of apples stored at 3° began on 21st October, 1929, that of the second on 6th January, 1930, and that of the third on 22nd April, 1930. The apples stored at 10° were tested on 2nd February, 1930.

Table III.

Dose g. daily	Normal Bramley's Seedling 1929 Stored 3° 1st batch	Normal Bramley's Seedling 1929 Stored 3° 2nd batch	Normal Bramley's Seedling 1929 Stored 3° 3rd batch	Normal Bramley's Seedling 1929 Stored 10°
3	+ + + +	+ + + +	+ + + +	+ + +
5	+ + + +	+ + + +	+ + + +	+ + + +
10	+ + + +	+ + + +	+ + + +	+ + + +
20	Test not finished but fully protected for 60 days	+ + + +	+ + + +	+ + + +

The results obtained in this experiment (Table III) yielded no definite answer as to whether the greater deterioration in the antiscorbutic activity of apples in "gas" storage was due to the temperature or to the composition of the atmosphere, since the slightly lower activity of the sample of apples stored at 10° for about 3 months as compared with that of the samples stored at 3° was of an order which falls within the limits of the experimental error of the

method employed in the assessment of vitamin C. It is of interest to note, however, that no loss of activity was recorded in Bramley's Seedling apples stored for 6 months at 3°.

The distribution of vitamin C in various parts of the apple.

In all the experiments of the preliminary and of this investigation the cortex of the peeled apples was used for the determination of the relative activity of the fruit. Experiments are here described in which the distribution of the vitamin in the fruit, carried out on some of the 1929 apples discussed in the preceding sections, is studied.

Three sections of the apples were tested, the peel, the outer half of the cortex and the inner half of the cortex of the peeled apples. The results are presented in Table IV. As it was not possible to use the usual number of 6 guinea-pigs, the number of animals employed in each test is noted in the table.

Table IV.

Normal Bramley's Seedling 1929. Stored 3°, 1st batch

Dose g.	Peel	No. of guinea-pigs	Outer cortex	No. of guinea-pigs	Inner cortex	No. of guinea-pigs	Total cortex from peeled apples	No. of guinea-pigs
½	+ + ±	4	Not tested	—	Not tested	—	Not tested	—
1	+ + + +	4	,,	—	,,	—	,,	—
1½	Not tested	—	±	3	,,	—	,,	—
3	+ + + +	4	+ + + +	2	+ ±	3	+ + + ±	6
5	Not tested	—	+ + + +	3	Vitiated test	—	+ + + ±	6
10	,,	—	Not tested	—	,,	—	+ + + +	6
20	,,	-	,,	—	Not tested	—	Test not finished but fully protected for 60 days	6

Normal Bramley's Seedling 1929. High nitrogen sample

Dose g.	Outer cortex	No. of guinea-pigs	Inner cortex	No. of guinea-pigs	Total cortex from peeled apples	No. of guinea-pigs
½	Not tested	—	Not tested	—	Not tested	—
1	,,	—	,,	—	,,	—
1½	+ +	3	,,	—	,,	6
3	+ + + +	3	+ +	3	+ + + ±	6
5	+ + +	3	+ + +	3	+ + + +	6
10	Not tested	—	+ + + +	3	+ + + +	6
20	,,	—	Not tested	—	Test not finished but fully protected for 55–60 days	6

	Frozen immature Bramley's Seedling 1929				Newton Wonder 1929			
Dose g.	Peel	No. of guinea-pigs	Total cortex from peeled apples	No. of guinea-pigs	Peel	No. of guinea-pigs	Total cortex from peeled apples	No. of guinea-pigs
½	+ ±	6	No protection	6	No protection	4	Not tested	—
1	+ + + ±	6	±	6	±	4	,,	—
1½	Not tested	—	Not tested	—	Not tested	—	,,	—
3	,,	—	+ + +	6	+ + + +	4	±	6
5	,,	—	+ + + +	6	+ + + +	4	+ ±	6
10	,,	—	+ + + +	6	Not tested	—	+ + + +	6
20	,,	—	Not tested	—	,,	—	Not tested	—

The tests began at the following times.

Normal Bramley's Seedling apples, 1929, at 3°: peel October 2nd, 1929, outer cortex January 6th, 1930, inner cortex November 22nd, 1929.

Normal Bramley's Seedling apples, 1929. High nitrogen: outer cortex January 14th, 1930, inner cortex November 22nd, 1929.

Peel of frozen, immature Bramley's Seedling apples, 1929, on August 8th, 1929.

Peel of Newton Wonder apples, 1929, on 3 g. and 5 g. dose, October 15th, 1929, and on 0·5 g. and 1 g., November 13th, 1929.

It will be seen (Table IV) that in the case of the normal Bramley's Seedling apple in which the potency was determined in the peel, in the outer cortex and in the inner cortex, the peel is more than 3–4 times as active as the outer cortex, and over 6 times as active as the inner cortex and that the outer cortex, as might be expected, is somewhat more active than the total cortex of the apple. In the case of the immature Bramley's Seedling and of the Newton Wonder, the relationship of the activity of the peel to that of the total cortex of the apples is similar to that of the normal Bramley's Seedling.

In this connection it should be mentioned that Givens, McCluggage and Van Horne [1922] have found dried apple peel more active than dried apple. Scheunert [1930] also states that the vitamin activity of apples depends on the method of peeling.

It is impossible to say at this stage of the enquiry whether the disparity in activity between the different parts of the fruit has any physiological significance, but in view of the similar distribution of certain enzymes in the apple such a possibility must be borne in mind.

Summary.

1. Newton Wonder apples possess a vitamin C content of the order of that possessed by Cox's Orange Pippin, whilst Lane's Prince Albert occupies an intermediate position between the latter and the much more potent Bramley's Seedling.

2. Bramley's Seedling apples can be frozen at − 20° and stored at this temperature for 4 months without losing appreciably in antiscorbutic activity.

3. Bramley's Seedling apples gathered at the end of July are not more active per g. than the normal apple from the same plantation gathered in October.

4. Bramley's Seedling apples stored at 3° in air for 5 months do not lose any of their antiscorbutic activity.

5. The concentration of vitamin C in the tissue of the apple increases as the skin is approached from the core and is more than 6 times as great in the peel as in the flesh near the core.

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XIX. THE ANTISCORBUTIC POTENCY OF APPLES. III.

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In comparing the potency of a number of varieties of apples, a particularly striking disparity between the antiscorbutic activity of the Bramley's Seedling and the King Edward was observed [Bracewell, Hoyle and Zilva, 1930, 1, 2] in spite of the fact that these two apples resemble each other in so many respects. A few chemical analyses disclosed also a difference in their nitrogen content. Thus, three nitrogen determinations yielded the following percentages: Bramley's Seedling 0·027, 0·035 and 0·038, King Edward 0·061, 0·070 and 0·072. In view of these indications it became necessary to establish definitely whether a relationship existed between the nitrogen content and the antiscorbutic activity of the apple. In the experiment here described an attempt was made to throw some light on this subject. The aim was to investigate Bramley's Seedling and King Edward apples grown under conditions which would conduce to the production of fruits of high and low nitrogen content similar to the above in both varieties. The following are the cultural details of the experimental material.

King Edward VII.

High nitrogen sample. The fruits were grown at Long Ashton Research Station. The soil, derived from the Keuper Marl formation, is a medium loam containing high proportions of fine sand and silt. The trees were planted in 1920, are under clean cultivation and have received annual dressings of fertiliser as under:

Nitrate of soda	at 2	cwt. per acre
Superphosphate	,, 3	,,
Sulphate of potash	,, 1	,,

The trees are of the bush type, are extremely vigorous and growth is very strong. They are just beginning to come to the age of bearing crops.

The 1929 crop was very light and all the fruits were large and of the vigorous type. The fruits were picked on October 8th, 1929.

[1] Member of the Scientific Staff, Medical Research Council, working with grants for assistance and expenses.

Low nitrogen sample. These were grown at Bratton, Wilts. The soil is derived from the Chalk formation and is a typical, thin, chalky soil. The trees were planted in 1922 and are of the bush type. They have only made medium growth. Since 1927 they have been grown under a sward of clover which has reduced vigour and resulted in typical "low nitrogen" trees. Potash manuring has been the manurial treatment given.

In 1929 vigour of growth was low, the leaves were yellowish green, the fruits small, with pale yellow ground colour and usually a fair reddish flush denoting low nitrogen conditions. The crop was heavy and the fruits were picked on October 8th, 1929.

Bramley's Seedling.

High nitrogen sample. These fruits were grown at Faversham, Kent. The soil is of mixed origin, being derived from surface deposits overlying chalk, probably largely Brick Earth and Thanet Sands material. It has the texture of a medium loam, containing high amounts of fine sand and silt fractions. The trees are about 20 years old, large and of the bush type, and are extremely vigorous in growth. Cultivation has been on a high level ever since planting. Fertilisers containing nitrogen and phosphorus have been freely applied but potash manures have only been applied to any extent during the last few seasons.

In 1929, vigour of growth was strong and the fruits were large, green specimens, being typically those of high nitrogen trees. The crop was heavy. The fruits were picked on October 9th, 1929.

Low nitrogen sample. These were also grown at Faversham, Kent, in an orchard situated near that on which the high nitrogen sample fruits were grown. The soil is of similar origin to that of the high nitrogen sample but is heavier and the site is rather drier. The trees are of similar age and type to those from which the high nitrogen fruits were taken but have always been less vigorous, and the vigour has been greatly checked by substituting grass culture for arable conditions.

In 1929, the trees were showing all the symptoms of severe nitrogen starvation, *i.e.* lack of shoot growth, very sparse, small and yellow leaves and small fruits of extremely high colour. The crop was heavy. The fruits were picked on October 9th, 1929.

On analysis the following percentages of nitrogen were found: *King Edward.* High nitrogen 0·0390 and 0·0387; low nitrogen 0·0305 and 0·0309. *Bramley's Seedling.* High nitrogen 0·0404 and 0·0372; low nitrogen 0·0166 and 0·0172. The high and low nitrogen figures of both varieties did not, unfortunately, coincide with the corresponding high nitrogen figures of King Edward and the low nitrogen figures of the Bramley's Seedling previously tested. In fact, the high nitrogen King Edward in this experiment contains about 30 % or 40 % less nitrogen than the apples of this variety used in the first experiment, whilst the nitrogen content of the high nitrogen Bramley's Seedling is

of the same low order shown by these apples in that investigation. There is, however, a definite difference in the nitrogen content in both cases.

The difference in the antiscorbutic activity between the high and low nitrogen Bramley's Seedling (Table I) falls within the limits of the experimental error of the method of the determination of vitamin C. The low nitrogen King Edwards are, on the other hand, definitely (about 1·5 times) more potent than the high nitrogen apples.

Table I.

These tests began on October 27th, 1929.

Dose g. daily	Bramley's Seedling 1929 Low nitrogen	Bramley's Seedling 1929 High nitrogen	King Edward 1929 Low nitrogen	King Edward 1929 High nitrogen
3	+ + + +	+ + + −	−	No protection
5	+ + + +	+ + + +	+ −	−
10	+ + + +	+ + + +	+ + + −	+ + −
20	Test not finished but fully protected for 55–60 days	Test not finished but fully protected for 55–60 days	+ + + +	+ + + +

These results, however, offer no clear-cut information for or against the theory that the nitrogen content of the apple has a bearing on its vitamin C content and consequently further attempts are being made in the hope of ascertaining whether such a relationship exists.

Summary.

King Edwards containing about 0·0307 % of nitrogen were about 1·5 times as potent antiscorbutically as apples of this variety containing about 0·0387 % of nitrogen. No significant disparity in the vitamin C content was found between Bramley's Seedlings containing high and low quantities of nitrogen.

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XX. THE BIOLOGICAL OXIDATION OF CARBOHYDRATE SOLUTIONS.

PART I. THE OXIDATION OF SUCROSE AND AMMONIA IN SECTIONAL PERCOLATING FILTERS.

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The manufacture of sugar from the sugar beet gives rise to large volumes of waste water of a polluting nature. About five-sixths of the beet sugar factory effluent originates from the water which is used to wash and convey the beet from open storage silos or from trucks into the factory. Under usual conditions the conveying or fluming water requires only simple treatment so that it may be used again in the factory. The remaining one-sixth of the total effluent is obtained from the beet pulp, which after extraction of sugar by diffusion, is dried and used as cattle food. Before the spent pulp is dried, as much water as possible is squeezed out of it. A "press water" is thus obtained which is highly polluting by reason of dissolved organic substances. Numerous attempts have been made on the Continent to devise a process which would destroy the dissolved substances but the methods tried have not been attended with great success.

Press water is a liquid of varying composition, but to illustrate its nature the following analysis of a sample taken from a factory is given; sucrose 230, protein 23, pectin 4·3, pentosan 13·4 and saponin 0·2 parts per 100,000. In view of the pollution already experienced and the increase that would probably follow as a result of the extension of the beet sugar industry, investigations were begun in 1927 at the Rothamsted Experimental Station and at the Colwick factory of the Anglo-Scottish Beet Sugar Corporation, Nottingham, for the Department of Scientific and Industrial Research with the object of ascertaining whether liquids such as press water could be satisfactorily purified by biological oxidation on percolating filters. The results of the semi-commercial scale experiments at Colwick have already been briefly described in the first three annual reports of the Water Pollution Research Board of the Department of Scientific and Industrial Research [1928, 1929, 1930]. At Rothamsted preliminary experiments showed that effective oxidation of solutions of the approximate composition of press water could be achieved, after dilution, by filtration through biological or percolating filters. The filters consisted of earthenware drainpipes 24 inches deep and 6 inches diameter filled with 1/8 to 1/4 inch clinker. In order to obtain artificial press water, sliced mangolds

or sugar beet were first boiled with water, the sugar extract strained off, and the residual pulp was then squeezed in a press. The runnings were diluted to the desired strength. Such artificial press liquors were most effectively oxidised when diluted so as to contain 0·1 % of sucrose, and at this strength they were allowed to trickle continuously on to the filters at a rate of 4·6 cc. per minute, equivalent to 100 gallons per cubic yard of filter per day. In this manner 90–95 % of the sugar was oxidised.

It was observed that the mangold or beet extracts rapidly became acid, owing to the fermentation of the sugar. Thus the p_H value of the freshly made solutions fell from 7·5–8·0 to 3·0–5·0 in less than 24 hours. The effluent obtained from the filtration of such an acid solution, however, was invariably neutral or slightly alkaline. When the acid fermented solutions were made alkaline with milk of lime and then filtered, the activity of the micro-flora seemed to be impaired in some way and an effluent of inferior quality resulted. In later experiments it was found that when a solution made from beet press water was fermented for 8 hours at 35°, the products were approximately 45 % unchanged sugar, 40 % hydroxy-acids and 15 % aliphatic acids. The behaviour on filtration of solutions composed of sugar and such typical products of fermentation as acetic and lactic acid was therefore studied in some detail. The results of these experiments proved conclusively that the course taken by natural fermentation provides a suitable liquid for oxidation on percolating filters; a mixture of sugar, acetic and lactic acids derived from a fermented solution of sugar is more easily oxidised than the original sugar solution. Only a partial conversion of sugar to acid is desirable. It seems that the acids alone do not provide the filter organisms with the most suitable food material to encourage the growth of a highly efficient biological film; the acids are then only partially oxidised. Moreover, filters fed with either acetic or lactic acid require added nitrogen, whereas those provided with a mixed diet of sugar and acid appear able to utilise very small amounts of nitrogen or possibly to fix atmospheric nitrogen. An interesting observation made by Dr Sandon, of the Rothamsted Experimental Station, was that all the filters, whether fed with sugar, sugar plus acetic acid, sugar plus lactic acid, lactic acid, acetic acid or any similar combination with different forms of added nitrogen, seemed to support the same protozoan and insect population.

One of the most significant observations made during the 1928 experiments was the presence of nitrates in incompletely oxidised effluents. These experiments were carried out with the following solutions: (*a*) 0·1 % sugar with 0·003 % of nitrogen added as ammonium chloride, and made up in tap-water containing 0·0005 % of nitrogen as nitrate; (*b*) as (*a*) but with 0·003 % of nitrogen added as albumin. The effluents regularly contained 0·0015 % of nitrogen as nitrate when as much as 0·01 % of sugar was present at the same time. There was thus some evidence that nitrification may occur in presence of organic matter. This conflicts with the generally accepted view in sewage purification that oxidised nitrogen does not appear in an effluent until the

organic matter has been decomposed. Oxidation of carbon precedes that of nitrogen when solutions of organic nitrogenous compounds are stored in bottles and supplied with an ample quantity of oxygen. With asparagine, for instance, Adeney [1908] found that hydrolysis first takes place, giving aspartic acid and ammonia. This is followed by complete oxidation of the aspartic acid before the ammonia is nitrified. Although the results of previous investigations on the oxidation of dissolved substances have been confirmed, the generally accepted view that easily oxidisable carbonaceous matter inhibits nitrification on percolating filters was not supported by the results obtained with drainpipe filters. The frequent presence of nitrate and sugar in the laboratory filters might conceivably be explained by assuming that some of the sugar in the fresh liquor always side-tracked down the filter or ran straight through the medium and that such solution would form one of the numerous integral parts of the whole effluent. This possibility is dealt with in the present paper.

In some earlier experiments on biological filtration in 1927, mangold extracts containing 0·1 % of sucrose were run through drainpipe filters 2 feet deep. The disappearance of organic matter was measured at depths of 6, 12, 18 and 24 inches by means of the oxygen taken up by the sample from acid permanganate in 4 hours, with the result that the upper half of the filter removed the greater part of the organic matter just as in sewage filters. It is apparent that where the food supply is richest and the air supply is plentiful, micro-organisms will become more abundant. They are then able to utilise the strong solutions which are run on to them. Moreover, with liquids such as sewage, the bacteria and zooglea can coagulate colloidal or suspended organic matter and then live on the coagulum. The lower part of a biological filter is thus apparently inefficient and contains less vital film only because its food supply is limited by the activity of the upper part.

The work described in the present paper was undertaken with the following objects:

(1) to account for the advantage of oxidising a solution of sugar partly fermented to acids over that of an unchanged solution of sugar;

(2) to follow the course of sugar decomposition and nitrification of ammonia through different levels of a percolating filter;

(3) to ascertain the function of different sections of a percolating filter.

Experimental.

Apparatus and methods of analysis.

Apparatus (Fig. 1). The sectional filter used in these experiments consisted of 6 glass cylinders each 6 inches in diameter and 9 inches deep. The cylinders rested in perforated zinc trays supported on the ledges of a vertical frame. Liquid passing through the perforations was caught on a funnel and then dropped on to the next section. In order to obtain a constant flow of sugar

solution through a tube wide enough to be cleaned easily of bacterial growths, the apparatus shown in Fig. 1 was used. This device controls the hydrostatic head of solution in an aspirator by means of a length of fine capillary tubing. The sections were filled with 1/8 to 1/4 inch clinker which had been well washed after use in similar filtration experiments.

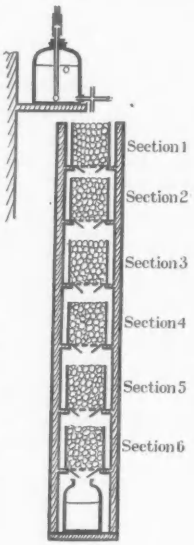

Fig. 1. Sectional percolating filter.

Methods of analysis. Sucrose. (*a*) This was determined by the Hagedorn-Jensen method as modified by Hanes [1929]: 2·5 cc. of the solution were inverted with 1 cc. *N* H_2SO_4 by heating on a boiling water-bath for 20 minutes and then neutralised with 1 cc. of *N* NaOH. The sucrose was returned as glucose. The limits of the Hanes modification are from 1·74 to 4340 mg. glucose per 100 cc.

(*b*) An alternative modification has been used for the range of 0·347 mg. to 3470 mg. glucose per 100 cc. A solution is made up containing 3·3 g. potassium ferricyanide and 10·6 g. sodium carbonate per litre. 10 cc. of this solution and from 0·1 to 10 cc. of the unknown solution are heated according to the usual procedure and the residual ferricyanide is finally determined by reducing it with KI and titrating with *N*/200 $Na_2S_2O_3$.

Invert sugar. The Hagedorn-Jensen solutions were used but 10 cc. of the ferricyanide solution were taken instead of 2 cc. and the volume of invert sugar made up to 10 cc. The range of the method is thus greatly increased, the difference between the blanks on successive days is also reduced and more reliable results are obtained than in the original Hagedorn-Jensen method. While the limits of the latter method are from 0·868 mg. to 347 mg. glucose per 100 cc. those of the modified method are from 0·174 mg. to 1735 mg. per 100 cc.

Nitrogen as NH_3. (*a*) Whenever the volume of solution permitted 250 cc. were distilled with ammonia- and nitrogen-free NaOH made according to the Ministry of Health Methods of Analysis [1929, p. 20]. The distillate was titrated with *N*/25 H_2SO_4 using cochineal as indicator.

(*b*) When the above method was impracticable 50 cc. of the solution were evaporated to dryness in a water-bath and the residue was taken up in 5 cc. *N* NaOH and 50 cc. 95 % alcohol. This solution was then steam-distilled in Foreman's apparatus [1928] until 100 cc. of distillate were collected and the ammonia was titrated with *N*/25 H_2SO_4. It was found that this method gave a complete recovery of ammonia from dilute solutions of ammonium salts.

Nitrogen as NO_2. This was determined colorimetrically as described in the Ministry of Health Methods [1929, p. 8].

Nitrogen as NO_3. The residue in the steam distillation flask left after the determination of ammonia by Foreman's method was neutralised with normal acid and made up to 200 cc. One-half of this solution was reduced by incu-

bation overnight at 35° with a zinc-copper couple; the ammonia was then distilled off and estimated by nesslerisation. The remaining half of the solution was incubated without the addition of a reducing agent, distilled and the distillate nesslerised. This represents the blank on the method. Details of the method are given in the Ministry of Health Methods [1929, p. 11].

Oxygen absorbed from permanganate in 4 hours. 10 cc. of $N/8$ $KMnO_4$ and 10 cc. of 25 % H_2SO_4 were incubated for 4 hours at 35° with sufficient solution of sugar or effluent to reduce not more than one-half of the permanganate. The unchanged permanganate was estimated with $N/20$ sodium thiosulphate. The amount of permanganate reduced is practically proportional to the sugar in the solution, provided the quantity of permanganate decomposed remains constant.

Biological oxygen demand, i.e. *the oxygen absorbed from solution in 5 days or 20 days.* In order to carry out this determination the dissolved oxygen present in a mixture of tap-water and the solution under examination is found. The mixture is then incubated for either 5 or 20 days at 20° and the oxygen absorbed from solution in the oxidation of the organic matter is obtained from a second oxygen determination. Under rigidly controlled conditions the oxygen taken up is approximately proportional to the organic matter present in the diluted solution.

p_H *value.* This was determined with the Hellige apparatus. The figures obtained gave good agreement when checked by the quinhydrone electrode method.

Total nitrogen. Before the solution was analysed as in the Kjeldahl method the nitrates and nitrites present were first reduced in the way described under *Nitrogen as* NO_3.

Experiments.

Exp. 1. Filtration of a mixture of sucrose, and acetic and lactic acids. A solution composed of 0·033 % sucrose, 0·033 % acetic acid and 0·033 % lactic acid was made up in Harpenden tap-water and run on to the sectional filter at a rate of 9·0–9·5 cc. per minute. For the first 42 days the removal of organic matter from solution was measured only by the oxygen absorbed from permanganate and the biological oxygen demand tests. Adequate information on the rate of maturation of these filters had already been obtained and this information will shortly be published; hence no detailed analyses were made in the early stages of the experiment. The experiment proved that the acids were rapidly oxidised in the first and second sections. Thus the p_H of the original mixture which was 4·65 increased to 6·0 in the first section and 6·5 in the second; it then increased uniformly until the liquid was discharged from the sixth section at a p_H of 7·5. It should be noted that the nitric nitrogen in the final effluent during this period never exceeded 0·2 part per 100,000, although in the tap-water used to make up the nutrient solution there was 0·5 part of nitric nitrogen present.

Exp. 2. Filtration of a solution of sucrose and ammonium chloride. The further filtration of sucrose, and acetic and lactic acids was postponed on the 43rd day until suitable methods for the exact analysis of the substances in dilute solutions were available. From that day until the 88th day the following solution was fed to the filter at a rate of 9·5 cc. per minute.

Sucrose	1·0 g.
NH_4Cl	0·1528 g. (increased on 120th day
K_2HPO_4	0·002 g. to 0·191 g.)
$MgSO_47H_2O$	0·0006 g.
NaCl	0·0002 g.
$CaCl_26H_2O$	0·1375 g.
Fe_2Cl_6	Trace
Distilled water	1 litre

C/N ratio until 120th day = 10/1: thereafter 8/1.

Analyses were made every few days over a period of 45 days and the results obtained are given in Table I.

Table I. *Percentage of sugar oxidised.*

Section ...	1	2	3	4	5	6
By 4 hours' test	22·5	39·0	46·0	53·2	58·4	67·7
By 5 days' test	25·2	42·6	44·4	50·2	56·5	65·5

The nutrient solution was made up daily and adjusted to a p_H value of 7·0.

As the filter began to mature a drop in p_H of the effluent from the first section indicated that acid was being produced in this part of the filter. For example, on the 58th day of the experiment the p_H figures were:

Fresh liquid	Section 1	2	3	4	5	6
7·0	6·6	6·9	7·3	7·3	7·4	7·4

Similar observations have previously been made in the Microbiology Department at Rothamsted.

The p_H figures given were obtained during about the first half hour, at the commencement of each day's run. Thereafter the p_H of the fresh liquid fell progressively during its 24 hours' storage. Even by careful sterilisation of the aspirator it was difficult to prevent the p_H of the nutrient solution from falling below 5·5 to 6·0 at the end of the day. As a rule, however, the aspirator was cleaned out daily with distilled water and every few days with hydrochloric acid. Under these conditions a liquid of p_H 6·5 or less was filtered for the greater part of the day and there was no measurable production of acid. Typical examples are given in Table II.

Table II. p_H *of nutrient solution and sectional effluent.*

Sucrose solution	Section 1	2	3	4	5	6
6·5	6·8	7·3	7·4	7·4	7·4	7·4
5·5	6·6	6·9	7·0	7·1	7·2	7·2
4·6	6·4	6·8	7·0	7·1	7·2	7·2

Nitrogen assimilation. The nitrogen supplied as ammonium chloride was assimilated by the micro-organisms present in all the sections, but by those in No. 1 more than any other. It was evident from inspection that No. 1 section contained the bulkiest film. Compared with the other sections it oxidised the largest proportion of sugar. This is clearly shown in Table III.

Table III. *Nitrogen present in nutrient solution and effluents.*

Parts per 100,000.

Day of experiment	Nutrient solution	Section 1	2	3	4	5	6
54th	4·000	3·752	2·960	2·738	—	—	1·949
73rd	4·000	2·744	2·632	2·464	2·464	2·072	1·624
86th	5·000	4·704	3·472	—	3·248	—	2·688
112th	4·000	3·892	3·612	3·416	3·258	2·940	2·310
122nd	5·000	4·021	3·789	3·428	3·092	2·890	2·576
128th	5·000	4·372	4·230	—	—	—	—

It is apparent that nitrogen is continuously removed from the solution. This does not always signify an increase in vital film and therefore a correspondingly greater percentage oxidation of sugar; nitrogen may be lost by the decay of the organic film and its discharge as humus or by the removal of living micro-organisms in the final effluent or by the breeding and growth of flies on the filter, as rapidly as it is assimilated as ammonia.

Nitrification. In proportion to the amount of nitrogen locked up by the filter the quantity of nitrogen oxidised to nitrous and nitric acid is quite considerable. There appears to be a fine balance between the amount of nitrogen assimilated and carbohydrate oxidised on the one hand, and the amount of nitrogen oxidised. Where the film is thick and air passage somewhat restricted as in the upper sections, carbohydrate combustion is vigorous and nitrogen assimilation is appreciable. But in the lower sections, where organic matter is present in low concentrations (from 0·01 to 0·05 % sucrose) the filter is specialised for nitrification. It will be seen from Table IV, however, that

Table IV. *Nitrification in presence of ammonia and sucrose.*

	86th day Rate 9 cc. per min.				99th day Rate 5 cc. per min.				112th day Rate 5·85 cc. per min.			
	% sugar	N present as			% sugar	N present as			% sugar	N present as		
Section	oxidised	NH_3	NO_2	NO_3	oxidised	NH_3	NO_2	NO_3	oxidised	NH_3	NO_2	NO_3
1	9·4	4·66	0·01	0·03	10·8	5·0	0·01	0·0	37·6	3·89	0·05	0·17
2	9·7	3·43	0·01	0·03	24·0	4·3	0·01	0·12	4·4	3·61	0·05	0·20
3	6·7	—	0·01	0·03	5·3	4·2	0·02	0·24	6·3	3·42	0·05	0·29
4	8·7	3·17	0·01	0·07	28·6	3·0	—	—	9·9	3·26	0·06	0·32
5	10·7	2·08	0·01	0·15		2·5	0·03	0·25	8·3	2·94	0·07	0·37
6	22·0	2·57	0·02	0·10	6·6	—	—	0·52	11·6	2·31	0·05	0·61
Total	66·2				75·3				78·1			

complete absence of carbohydrate is not essential before nitrification can occur. On the 112th day, for instance, the effluent from Section No. 4 contained 44 parts of sucrose per 100,000, 0·06 part of nitrogen as nitrite and

0·32 part of nitrogen as nitrate. The solution passing through the filter receives a thorough mixing and it is therefore highly improbable that a small volume of nutrient solution passes through four sections and escapes oxidation while the rest of the solution undergoes a more complete oxidation. In other words, bacteria capable of oxidising ammonia to nitrite and nitrite to nitrate are actively present in a solution which contains free ammonia and carbohydrate.

Exp. 3. Interchange of sections Nos. 1 and 2. The effect of interchanging section No. 1 with No. 2 was studied from the 129th day of the experiment to the 157th. While section No. 2 showed a rapid increase in efficiency, due to the fact that it received a stronger nutrient solution, No. 1 fell off slowly. The net effect of the change, therefore, is to increase the capacity of the two upper sections to oxidise sugar. This is shown in Table V.

Table V. *Percentage of sugar oxidised.*

	Percentage of sugar oxidised.						
Section	Day before change	1st day of change	3rd day of change	6th day of change	14th day of change	20th day of change	29th day of change
No. 1	32·1	10·7	24·2	22·9	26·9	32·2	40·3
No. 2	8·0	34·4	35·9	24·4	21·4	17·0	26·8
No. 1 + No. 2	40·1	45·1	60·1	47·3	48·3	49·2	67·1
All sections 1 to 6	78 (about)	—	—	—	89·1	—	100·0

It was shown in Exp. 2 that the production of acid in Section No. 1 depends on the p_H of the nutrient solution. If this is less than 6·7 or 6·8 then increase in acidity cannot be detected. The interchange of Sections Nos. 1 and 2 gave additional evidence in support of the view that the p_H of the nutrient solution controls acid formation. Thus in Table VI it is seen that for 2 days after Section No. 1 had been placed in No. 2 position, it received a liquid of p_H 6·8. This section still retained its powers of acid production, for the p_H was lowered to 6·6. A few days after the change, however, it was unable to produce acid from a liquid of p_H 6·8.

Table VI also indicates the rapid oxidation of acid, even by an immature section such as No. 2.

Table VI. *Effect of interchange on* p_H *values.*

Day before change		1st day of change	2nd day of change	3rd day of change	6th day of change	14th day of change	29th day of change
Nutrient solution	6·0	6·3	6·8	6·0	4·4	6·0	6·6
Section No. 1	6·4	6·8	6·8	6·6	6·0	6·8	6·6
Section No. 2	6·4	6·6	6·6	6·8	6·4	6·9	6·8

Estimations of nitrate in the effluents of the sections interchanged were made at frequent intervals. These analyses are given in Table VII. It may be stated that the average amount of nitrate present in the effluent from the first section did not exceed 0·05 part N per 100,000, while in the second section this increased to 0·1 part. When the change of section was made nitrate was

definitely absent from the first effluent after the 6th day. The effluent from the next section (No. 1 temporarily in the place of No. 2) contained appreciable amounts of nitrate, though the results were somewhat erratic. However, sufficient nitrate was present to prove that nitrification had actually occurred in the presence of sucrose and invert sugar, equivalent in concentration to 0·06 to 0·07 % of glucose.

Table VII. *Nitrogen as nitrate.*

Parts per 100,000.

Day before change		Section	1st day of change	3rd day of change	6th day of change	14th day of change	29th day of change
Section No. 1	0·105	No. 2	0·095	0·056	0·024	0·0	0·0
Section No. 2	0·114	No. 1	0·125	0·111	0·048	0·378	0·200

Exp. 4. Interchange of Sections Nos. 1 and 6. In order to confirm the results obtained in Exp. 3, Section No. 6 was made the first section and No. 1 the last. This experiment brings out the effect of a 0·1 % sucrose solution on the biological activity of nitrifying organisms. It also demonstrates the reaction of a mixed biological flora to solutions of ammonia and nitrite in which little carbohydrate is present.

Turning to the consideration of the course of the sugar oxidation, it is seen that the oxidising power of Section No. 6 rapidly increased in its new position. Prior to the interchange the progressive removal of sucrose as the solution percolated through the filter was of the following order: Section No. 1, 40·3 %; No. 2, 26·8 %; No. 3, 6·8 %; No. 4, 11·8 %; No. 5, 12·0 %; No. 6, 2·3 %; total 100 %. When these figures are compared with those given in Table VIII, the development of active film in Section No. 6 is shown by the greater proportion of sugar removed by this section. Within one month it was as active as either Sections 1 or 2, when these received the fresh solution first.

Table VIII. *Percentage of sucrose oxidised.*

Day before change		Section	1st day of change	4th day of change	7th day of change	11th day of change	27th day of change
Section No. 1	40·3	No. 6 (now No. 1)	14·9	18·4	21·5	14·5	42·5
Section No. 6	2·3	No. 1 (now No. 6)	—	—	—	15·8	12·5

It is a remarkable fact that the filter, considered as a whole, did not suffer by the sudden change of conditions in this experiment. This is due partly to the fairly slow reduction in rate of oxidation shown by Section No. 1, partly to the efficiency of this latter section in the last position even after one month and partly to the increasing activity of Section No. 6 in the first position. That the overall efficiency of the filter was not impaired is seen from the following example of the percentage removal of sugar effected by the sections in the order they were present in the filter: Section No. 6, 42·5 %; No. 2, 13·9 %; No. 3, 14·9 %; No. 4, 9·0 %; No. 5, 1·7 %; No. 1, 12·5 %; total 94·5 %.

For the first few hours of the change of Sections Nos. 6 and 1, nitrite and nitrate were found to be present to the extent of 0·018 and 0·190 part N per 100,000 respectively in No. 6 effluent (temporarily No. 1); in No. 1 effluent (temporarily No. 6), the amounts were 0·036 and 0·230 respectively. Within one week nitrifying activity had almost ceased in No. 6, only 0·008 part of nitrogen as nitrite and 0·04 part as nitrate per 100,000 being obtained after 6 days. Section No. 1, however, became more active in this respect: the effluent contained 0·04 part of nitrogen as nitrite and 0·44 part as nitrate. There seemed to be a tendency for nitrate to appear suddenly in Section No. 6 and then disappear. Insufficient evidence exists on which to base any satisfactory explanation of this apparent anomaly.

Inversion of sucrose during filtration. When sucrose solutions were run on to non-sectional percolating filters only small amounts of invert sugar were found in the effluents. Analysis of samples taken at various levels of the sectional filter used in the previous experiments proved that a considerable fraction of the total sucrose was invariably changed into invert sugar. Typical results are given in Table IX.

Table IX. *Sucrose and invert sugar.*

Calculated as mg. glucose per 100 cc.

	102nd day of experiment		122nd day of experiment		157th day of experiment		185th day of experiment	
Sample	Sucrose	Invert sugar	Sucrose	Invert sugar	Sucrose	Invert sugar	Sucrose	Invert sugar
Fresh solution	108·02	1·10	112·34	0·86	130·9	—	111·50	—
Eff. No. 1	81·57	8·67	73·39	2·25	67·35	10·85	53·54	10·54
Eff. No. 2	68·04	9·88	56·88	4·83	33·28	9·72	37·90	10·75
Eff. No. 3	55·23	11·97	48·23	6·97	28·82	5·38	22·31	9·68
Eff. No. 4	37·66	15·26	36·15	11·15	10·44	8·26	15·26	6·73
Eff. No. 5	33·02	12·85	34·63	11·37	0·00	3·05	14·47	5·66
Eff. No. 6	35·25	11·75	32·11	11·69	0·00	—	4·07	2·34

It is seen from Table IX that appreciable amounts of invert sugar are present in certain sections of the filter. The quantity of invert sugar produced by any particular section does not remain even approximately constant during the development of the biological film. It is not improbable that the limiting factor in the oxidation of sucrose is the rate of oxidation of the invert sugar. Hence, when the filter was still immature (up to about the 130th day) invert sugar would tend to accumulate in the lower sections, owing to the inability of the organisms in these sections to decompose as much invert sugar as they produced. After the 130th day the filter reached maturity. The lower sections were then able to oxidise invert sugar *in situ* as rapidly as it was formed, in addition to part of that presented by the upper sections.

Discussion.

The experiments described in this paper prove that a biological film may be built up on a filter made up of sterile clinker by allowing a 0·1 % solution of sucrose and an adequate supply of available nitrogen to percolate slowly

through the filter. If the rate of flow of this nutrient solution is kept low and aerobic conditions are maintained, any desired degree of oxidation can be effected. Before the microbial population has become established the clinker removes a considerable proportion of the sugar by adsorption but once the organisms begin to develop loss of sugar due to purely physical forces becomes insignificant. The sugar is then oxidised entirely by the micro-organisms. When the part played by each section of the filter used for these experiments is examined, it should be remembered that the first section is given an advantage in the initial stages which becomes more pronounced as the filter matures. This advantage lies in the concentration of sugar in the nutrient solution which is run on to No. 1 section. Table I exhibits the beginning of the superiority of Section No. 1 over all other sections. As this section oxidised 25 % of the sugar, the second section can only decompose 18 % of the total sugar, assuming an efficiency equal to that of the first section. Each development of film in the top section therefore increases the difference between the amount of sugar oxidised by Section No. 1 and by lower sections. Further advantages which the top section gains are due to the fact that the water-holding capacity of the film and its absorptive power increase with the growth of film. The sugar therefore remains in contact with micro-organisms for a longer period in this part of the filter than in any other. A quantitative expression of the average time of contact of liquids with filter medium has already been worked out by an elaboration of Clifford's method [1907] and it has been shown that the time of contact might increase to two or three times the contact of the clean, sterile medium, as a result of film development. The sectional filter was not regarded as mature until it was able to oxidise 90 to 100 % of the sugar it received. The percentage of sugar oxidised by the different sections was then approximately: No. 1, 40 %; No. 2, 25 %; No. 3, 12 %; No. 4, 10 %; No. 5, 7 %; No. 6, 6 %.

Under suitable conditions each part of a filter is able to perform the work of any other section merely by interchanging the sections. An example of the operation of this principle is shown in Exp. 4. In this case the sixth section, which contributed very little towards the total oxidising effect of the whole filter, but was highly specialised for nitrification, was made the first section for one month. During this period microbial counts showed that there was a rapid development of yeasts, bacteria and protozoa in this section which corresponds with a parallel increase in the amount of sugar oxidised. At the end of the month as much sugar was completely oxidised to CO_2 and water by the sixth section (temporarily the first) as by the section previously in the first position. Even after one month, however, the latter section was able to decompose more sugar than the original sixth section and at the same time effect a considerable nitrification. It thus appears that a filter gains in efficiency when its various parts are matured by receiving in turn the strong nutrient solution.

The sequence of changes which the sugar undergoes in its ultimate

breakdown to CO_2 and H_2O is so far mainly a matter for speculation. A mixed flora exists on the filter and contains organisms which may bring about almost every known type of fermentation with the appropriate end products. In a mature aerobic filter the population co-operates in oxidising the sugar and only such organisms will exist in any quantity as can effectively make use of the sugar and of the waste products of other organisms. Intermediary products will thus tend to be oxidised as soon as they are formed. There is an alternative possibility that the usual products of fermentation are not formed to any extent under aerobic conditions of a percolating filter, or if formed are capable of rapid oxidation *in situ* by the organism which produces them. This idea gains in probability when it is remembered that typical products of bacterial fermentation are relatively stable in anaerobic cultures but break down to CO_2 and water when air is admitted to the cultures. Lactic acid, for example, accumulates in anaerobic yeast fermentations but disappears when the yeast is aerated [Fürth and Lieben 1922], or when the fermented liquid is treated on a percolating filter (unpublished work). There is little doubt, however, that a considerable proportion of the sucrose is first inverted as an intermediate stage in the oxidation. There is some evidence that an acid product is also formed, but the nature of this substance is as yet unknown. It has already been stated that the drop in p_H—evidence on which this claim is based—is recorded as the nutrient solution passes out of the first section. The latter is thick with film, which might restrict the passage of air and so produce partially anaerobic conditions and acid fermentation in those parts of the film not exposed to air.

Turning to a consideration of the role of nitrogen in the sectional filter, it is apparent that a constant supply of available nitrogen is necessary for the development and maintenance of the biological film; the greater the amount of active film the greater the demand for nitrogen. It has been shown in large-scale filters (unpublished work) that for reasons already stated the quantity of film steadily decreases from the top of the filter to the bottom. The results of the work described in this paper also show that there is a gradient of nitrogen assimilation, or protein synthesis, corresponding to the amount of film present in different levels of the filter. When an excess of nitrogen over and above this amount is added, nitrites and nitrates are produced. These compounds appear in effluents from the middle and lower sections of the filter even in the presence of appreciable quantities of sugar. For example, pronounced nitrification has occurred over long periods in solutions containing up to 60 parts of sucrose per 100,000. Similar observations were made two years ago when, as previously stated, the filter was not divided into sections. Since the work of Winogradsky [1891], it has been held that small concentrations of organic substances inhibit or prevent nitrification. This belief has repeatedly been attacked, as, for instance, by Beijerinck [1914]. There is, in fact, much conflicting evidence in support of either view. Until recently no satisfactory explanation of these contradictory facts could be

given. Cutler [1930], however, has reported that a number of organisms capable of producing nitrite from ammonia have been isolated from Rothamsted soil and percolating filters similar to the one described in this paper. Runov [1926, 1928] and Mischustin [1926, 1928], described nitrifying bacteria which multiply in media containing organic matter. The discovery of other organisms which transform nitrites into nitrates in the presence of organic matter therefore appears to await the solution of a suitable technique for their growth and isolation. The experiments described in this paper suggest the existence of such organisms.

SUMMARY.

1. The experiments described have been carried out with a percolating filter. This consisted of six independent sections, each 6 inches diameter and 9 inches deep, and filled with 1/8 inch to 1/4 inch clinker.

2. When a mixture of 0·033 % sucrose, 0·033 % acetic acid and 0·033 % lactic acid is fed to the filter described in (1) at a suitable rate of flow, it is readily oxidised by a mixed microbial population. A biological film is built up and the organic matter is oxidised even if the C : N ratio of the nutrient solution is 80 to 1.

3. If a 0·1 % solution of sucrose is allowed to trickle through a mature portion of the sectional percolating filter, some formation of acid and invert sugar takes place. For acid to be produced the p_H of the nutrient solution must not be less than 6·7 to 6·8. The maximum p_H drop recorded has been from an initial p_H of 7·0 to one of 6·6. The nature of the acid product is as yet unknown. It is suggested that the acid arises either as a normal intermediate product of sucrose oxidation which is destroyed almost instantaneously, or that its presence is due to the growth of a bulky film within which an acid, anaerobic fermentation occurs.

4. When the sectional filter was working well within its capacity, *i.e.* more than able to oxidise the organic matter with which it was supplied, it removed sucrose from a 0·1 % solution in approximately the following manner: Section No. 1, 40 %; No. 2, 25 %; No. 3, 12 %; No. 4, 10 %; No. 5, 7 %; No. 6, 6 %; total 100 %.

5. Each section of the percolating filter may be replaced by any other without the filter losing in efficiency. In fact the total working capacity of the filter could probably be increased by making each section the first in turn for a few weeks.

6. Oxidation of ammonia proceeds best in the lower sections where the concentration of sugar is least. It is not inhibited by concentrations of sugar up to 0·05 to 0·06 %. Therefore nitrification and carbohydrate oxidation take place simultaneously in several of the sections. Ample evidence exists in support of the view that bacteria which nitrify ammonia may be active in presence of organic matter.

7. Both nitrites and nitrates are produced by micro-organisms in the presence of 3 to 4 parts per 100,000 of nitrogen as ammonia.

8. Each section, irrespective of its previous function, rapidly shows nitrifying activity when placed in a suitable part of the filter.

The unpublished results referred to in the Introduction were obtained by Messrs E. H. Richards, R. B. Dawson, S. W. Johnson, J. T. Martin and the author. Mr E. H. Richards, Head of the Fermentation Department, has been personally responsible for the direction of the work described in this paper and his keen interest and stimulating criticisms have been highly valued.

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XXI. NOTES ON THE HAGEDORN-JENSEN METHOD FOR THE DETERMINATION OF BLOOD-SUGAR.

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(*Received December 3rd, 1930.*)

The Hagedorn-Jensen method for the determination of blood-sugar is frequently used not only in clinical blood analysis but also in research work. Most authors employing the method consider it quite satisfactory and accurate. Their criterion is the fact that the method yields very good duplicates. A few critical studies, however, cast some doubt upon its reliability. Thus, Duggan and Scott [1926] point out that since 0·01 cc. of 0·005 *N* thiosulphate is equivalent to 1·8 mg. of glucose per 100 cc. of blood, very slight differences in titration may cause considerable errors. This and the fact that the method employs 0·1 cc. of blood represent *a priori* limitations of accuracy. Folin and Malmros [1929] call attention to a more serious inherent source of error. They state that the cotton plug used for filtration, if too small and loose, allows some precipitate to pass through, rendering the results too high. If, on the other hand, the plug is larger and compact, it retards filtration and retains some filtrate, causing loss of sugar.

As a first step in our examination of the Hagedorn-Jensen method we performed numerous determinations, strictly following the original directions, on blood samples containing known amounts of glucose. These were prepared according to Somogyi [1930] by the addition of glucose to blood samples which were rendered sugar-free either by treatment with washed yeast or by glycolysis. Although duplicate determinations as a rule showed very good agreement, the quantities of the glucose recovered differed greatly from the amounts actually added. This points emphatically to the fallacy of the assumption that good checks between duplicate determinations may be considered as a criterion for the adequacy of a method. The criterion we have adopted in this work is the accuracy with which known amounts of glucose, added to sugar-free blood, can be recovered. The Hagedorn-Jensen method, as we shall presently show, proved to be entirely unreliable in such a test.

Next, we examined to what extent the source of error, pointed out by Folin and Malmros, may account for the faulty results. To determine the amount of sugar retained by the cotton plugs, after filtration and two washings,

[1] Working with a grant from the Selma Michael fund.

two or three plugs from parallel determinations were united and extracted with 12 cc. of water; the extract was filtered through a washed filter-paper, and the reduction of the filtrate was determined. Having found that cotton gives up reducing substances to water in measurable amounts, we employed in these experiments cotton which had been carefully washed and dried at room temperature. The results, given in Table I, show that 4 to 22 mg. per 100 cc. of the sugar is lost in the cotton filters, amounts of the same order of magnitude as Folin and Malmros found by a different technique.

Table I. *Showing that the cotton filters in the Hagedorn-Jensen method retain appreciable portions of the blood-sugar.*

No.	Blood-sugar in Hagedorn-Jensen filtrates mg. per 100 cc.	Blood-sugar retained in cotton filter mg. per 100 cc.
1	24	4
2	54	5
3	123	7
4	137	7
5	151	22
6	182	11
7	213	14
8	217	8
9	224	22
10	318	10

The magnitude of the loss depends largely upon the concentration of the blood sugar, assuming uniform bulk and density of the filter plugs. In the hands of one and the same worker the method may thus yield good duplicate determinations, while the results do not represent the actual total of the reducing substances.

Before carrying our investigations further we had to eliminate this source of error. To this end we used the Hagedorn-Jensen method in the following form. In a test-tube marked at 12 cc., 0·12 cc. of blood was precipitated according to the directions of Hagedorn and Jensen. After heat-coagulation the mixture was cooled and diluted to 12 cc. The test-tube was stoppered and well shaken, and the contents were filtered through dry filter-paper. Filter-paper thoroughly washed with slightly acidified water was used in this work. For the rest, the original procedure was followed using 2 cc. of the Hagedorn-Jensen ferricyanide reagent and 10 cc. of filtrate which corresponds to 0·1 cc. of blood. In order to prevent changes in the alkalinity of the reagent, the filtrates were always neutralised with 0·30–0·35 cc. of 0·1 *N* sodium hydroxide before the addition of the ferricyanide.

For the calculation of results we have prepared our own table with standard solutions of glucose. The original table cannot be used in accurate work, since Hagedorn and Jensen erroneously postulated that the self-reduction of ferricyanide reagents is always the same, and used a constant value for this as a correction. The fact is however that it is almost impossible to prepare two batches of Hagedorn-Jensen reagent that show the same self-reduction, and

the latter will change even in the same batch upon standing for some time. Instead we follow the procedure employed in other iodimetric methods and subtract the titration figures from the titration value of a "blank" sample of the reagent. The blank, in which water takes the place of the sugar solution, is treated exactly as the unknown. In this way self-reduction is ruled out and the difference value is equivalent to the reduction of the sugar solution. It is probably due to the arbitrary method of correction that Höst and Hatlehol [1920] also found the sugar table of Hagedorn and Jensen unsatisfactory and felt the necessity of preparing one of their own. In Table II are presented the basic figures of our table.

Table II. *Glucose equivalents of titration values in the Hagedorn-Jensen method.*

Actual amount of glucose mg.	0·005 *N* thiosulphate (blank titration) cc.	Blood-sugar if filtrate from 0·1 cc. of blood is used mg. per 100 cc.
0·01	0·07	10
0·02	0·12	20
0·05	0·26	50
0·10	0·53	100
0·15	0·80	150
0·20	1·07	200
0·25	1·33	250
0·30	1·60	300
0·35	1·87	350

Owing to the excellent proportionality between sugar concentration and reduction, a detailed table can easily be prepared by interpolation. Well reproducible results are obtained only up to a concentration of 350 mg. per 100 cc. blood-sugar; above this limit the values become unreliable. This difficulty we have obviated by increasing the concentration of the ferricyanide for the determination of higher concentrations of sugar. As to the lower limit, our experience agrees with that of Duggan and Scott [1926] who were unable to obtain good results below the 25 mg. per 100 cc. concentration of blood sugar (0·025 mg. of glucose).

In Table III are given examples of comparative sugar determinations by

Table III. *Showing that the original Hagedorn-Jensen method yields too low results for true blood-sugar.*

	True sugar* found Hagedorn-Jensen method		
No.	Original mg. per 100 cc.	Modified mg. per 100 cc.	Amount of added sugar mg. per 100 cc.
1	89	104	100
2	126	155	150
3	154	160	160
4	172	189	190
5	173	201	200
6	178	199	200
7	298	301	300
8	487	498	500

* True sugar = total reduction (apparent sugar)—reduction of sugar-free blood.

the original Hagedorn-Jensen method and by the technique eliminating the cotton filter. The figures represent true sugar values, obtained as the difference between total reduction and non-fermentable reducing substances. The latter were determined in the blood, after it had been rendered sugar-free by means of glycolysis or by treatment with washed yeast. To another portion of this sugar-free blood a known amount of glucose was added, and the total reduction was determined. The results obtained by the original Hagedorn-Jensen methods were computed from the original table of these authors, while those determined with our modification were read from our own table (Table II). As can be seen, the original Hagedorn-Jensen technique yields results that are too low, the discrepancies being rather irregular. From the figures of Tables I and III, it is apparent that the discrepancies are of the same order of magnitude as the losses of sugar in the cotton plugs, which shows that the technique of filtration is the essential source of inaccuracy, which renders the Hagedorn-Jensen method altogether inadequate for accurate analytical work. With the elimination of the cotton filter, and with a logical method of correcting for the self-reduction of the ferricyanide reagent, it is possible to obtain very satisfactory results.

This observation is entirely contradictory to all reports from other workers. Comparative blood-sugar determinations on record, carried out by the Hagedorn-Jensen method and several copper-reduction methods (Folin, Shaffer-Hartman, *et al.*) do not show appreciable or consistent differences in one direction or the other [Duggan and Scott, 1926; Van Slyke, Hiller and Linder, 1925; Höst and Hatlehol, 1920]. The apparent conflict however is readily explained by the fact that these authors determined apparent sugar values (total reduction), while we are dealing with true (fermentable) sugar. Somogyi has repeatedly pointed out [1930] that different reagents yield different values for the non-sugar reducing substances, and furthermore that different protein precipitants leave different quantities of these substances in the blood filtrates [Somogyi and Kramer, 1928]. In particular, ferricyanide gives much higher values for the non-fermentable reducing substances than do the copper reagents. We can corroborate the findings of Somogyi that the amount of the non-sugar reducing substances in the Hagedorn-Jensen method is 20–30 mg. per 100 cc., in the Folin-Wu method 12–15 mg. per 100 cc., in terms of glucose. The higher value in the Hagedorn-Jensen method obviously balances the loss of sugar in the cotton filter, thus concealing this serious source of error, and as a consequence the two methods frequently yield rather similar results. Comparison of total reduction instead of true sugar values, of course, could not disclose that fermentable sugar is actually lost in the Hagedorn-Jensen method.

In recent very careful experiments Herbert and Bourne [1930] found that the Hagedorn-Jensen method gives sugar values "slightly higher" than the true sugar obtained by Somogyi's method. The discrepancies vary between 1 and 10 mg. per 100 cc. According to our findings, such agreements are

possible only through coincidences in some cases in which the amounts of the reducing non-sugars, included in the Hagedorn-Jensen reduction values, happen to be the approximate equivalents of the amounts of sugar lost in the cotton filters. Since both the positive and the negative error are variable, the approach to true sugar values is bound to be quite inconsistent, and the discrepancies are frequently too great to be ascribed simply to "experimental errors."

SUMMARY.

An examination of the Hagedorn-Jensen method for the determination of blood-sugar reveals sources of error which render it inadequate for accurate work.

The method does not yield true sugar values, the equivalent of 20 to 30 mg. per 100 cc. of sugar being derived from reducing substances other than sugar. At the same time the results are considerably lower than would be expected, owing—as Folin and Malmros have shown before—to a retention of 4–22 mg. per 100 cc. of fermentable sugar in the cotton plugs employed for filtration.

The table of Hagedorn and Jensen, given for the computation of sugar, leads to an additional error. In this table a definite correction for the self-reduction of the alkaline ferricyanide reagent is applied, implying the assumption that self-reduction is a constant value for every reagent. A correction value of general validity however is impossible, as the self-reduction changes with time even in the same reagent.

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XXII. THE VAPOUR PRESSURE DEPRESSIONS OF AQUEOUS SOLUTIONS OF PHOSPHATE BUFFER MIXTURES AT 20·3°.

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(*Received December 18th, 1930.*)

The difficulties attendant upon the determination of the colligative properties of solutions, at temperatures other than the freezing and boiling points, have prevented the accumulation of data concerning these important physico-chemical functions. The direct measurement of osmotic pressure has been restricted almost entirely to a few organic substances, while accurate determinations of vapour pressure lowerings have been limited owing to the complexity of the methods and apparatus involved in the static and dynamic methods used heretofore. The recent introduction by Hill [1930, 1] of a thermal method for measuring vapour pressure, owing to its simplicity and ease of manipulation compared with the static or dynamic method, renders the determination of such colligative properties relatively easy. The method is particularly useful and accurate for the solution of many chemical problems, furnishing as it does a knowledge of a function whose thermodynamic relationship to other physico-chemical properties is well established.

The common use of Sørensen's [1912] phosphate mixtures as buffer solutions in biochemical work renders a knowledge of their colligative properties of great practical importance. Previous determinations of such properties have been limited to the freezing and boiling points [International Critical Tables, 1928] of solutions of the pure salts, no accurate data being available, even at these temperatures, for mixtures. Such data, therefore, at the suggestion of Mr G. S. Adair, have been obtained and are given below.

The vapour pressure lowerings recorded here were obtained by Hill's thermal method as described in previous papers from this laboratory [Hill, 1930, 1, 2; Margaria, 1930]. The same apparatus and general procedure were used as described elsewhere [Grollman, 1931]. The salts employed were standard "analytical reagents." The primary potassium phosphate (KH_2PO_4) was dried at 110° for a day before use. The secondary sodium phosphate ($Na_2HPO_4.2H_2O$) was obtained by exposing $Na_2HPO_4.12H_2O$ to the atmo-

[1] John Simon Guggenheim Memorial Fellow.

sphere, as suggested by Clark [1928]. The various buffer mixtures were prepared according to the usual procedures [Clark, 1928].

The results are given in Table I. Columns 1 and 2 of this table give the

Table I. *The vapour pressures of Sørensen's phosphate buffer mixtures at 20·3°.*

Composition of solution: Primary phosphate cc. of $M/15$ KH_2PO_4	Composition of solution: Secondary phosphate cc. of $M/15$ Na_2HPO_4	p_H	NaCl concentration isosmolar with solution. Moles per 1000 g. of water	Molar relative vapour pressure lowering
10·0	0·0	—	0·0637	$3·18 \pm 0·008 \times 10^{-2}$
9·5	0·5	5·589	0·0637	$3·18 \pm 0·013$
9·0	1·0	5·906	0·0639	$3·19 \pm 0·004$
8·0	2·0	6·239	0·0643	$3·21 \pm 0·016$
6·0	4·0	6·643	0·0657	$3·28 \pm 0·008$
4·0	6·0	6·979	0·0680	$3·39 \pm 0·015$
2·0	8·0	7·381	0·0698	$3·48 \pm 0·010$
1·0	9·0	7·731	0·0714	$3·56 \pm 0·013$
0·5	9·5	8·043	0·0737	$3·67 \pm 0·014$
0·0	10·0	—	0·0743	$3·70 \pm 0·016$

composition of the various mixtures investigated. Column 3 shows the p_H of such mixtures, according to Clark [1928]. In column 4 is given the sodium chloride concentration isosmolar[1] with the solution in question as found by experiment. Column 5 shows the relative molar vapour pressure lowering as defined by the expression $\frac{p_0 - p}{M p_0}$, in which p_0 is the vapour pressure of pure water at the temperature of the experiment, p the vapour pressure of the solution, and M the number of moles of solute present per litre of solution. The value of M is constant for the solutions studied and equal to 1/15. It should be noted that the above described mode of expression for the molar relative vapour pressure lowering differs slightly from that usually employed, in which M is expressed in terms of the number of formula weights of solute present per 1000 g. of water. The former mode of expression has been used because buffer solutions are generally expressed in terms of volume concentrations.

The vapour pressure lowerings for pure Na_2HPO_4 and KH_2PO_4 solutions, as given in the table, were obtained by comparison with a 0·064 molar NaCl solution whose molar relative vapour pressure lowering was taken as $3·33 \times 10^{-2}$, as previously found [Grollman, 1931]. The data for the mixtures were then obtained by comparison with the Na_2HPO_4 or KH_2PO_4 solutions. The values quoted are the averages of at least four sets of determinations (8 readings) and the probable errors of the mean molar relative vapour pressure lowerings are included in the last column.

It might have been expected that the vapour pressure lowering would be a linear function of the composition of the mixture. This would be the case were no interaction to occur between the constituent salts, *i.e.* were the total

[1] This term is used to describe a solution of the same vapour pressure as a given solution under the same condition of temperature, etc.

ionic activity of the solute to remain constant. This expectation is not fulfilled, as is seen in Fig. 1.

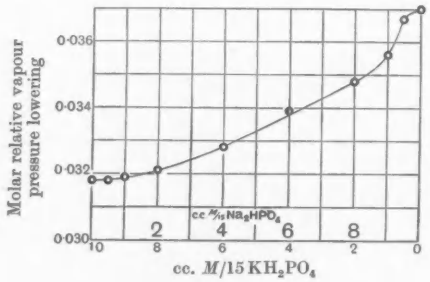

Fig. 1.

Ordinate. The relative molar vapour pressure lowering $(p_0 - p)/Mp_0$, where p_0 is the vapour pressure of pure water, p the vapour pressure of the solution, and M is the number of g. formula weights of solute per litre of solution.

Abscissa. The composition of the solutes expressed as cc. of $M/15$ Na_2HPO_4 in 10 cc. of the mixture.

It is often assumed in biochemical and physiological work that the colligative properties of mixtures, such as are continually encountered in biological materials, are simple additive properties of the constituents. The inadequacy of this view, even where no apparent interaction occurs between the various solutes (in which case, of course, no such additive relationship would be expected to hold), has been shown elsewhere [Grollman, 1931]. The curve of Fig. 1 emphasises again the extent of the deviation of the colligative properties of a mixture from those anticipated on the assumption of a simple additive relationship between the properties of the mixture and those of its constituents.

The vapour pressure values quoted in the present paper, being functions of the total ionic activities of the solutions, may be utilised in calculations involving expressions for such activities.

Summary.

Data are presented for the vapour pressure depressions of $M/15$ aqueous solutions of KH_2PO_4, of Na_2HPO_4, and of mixtures of these salts.

I am indebted to Professor A. V. Hill, at whose suggestion these measurements were made, for his constant interest and help. The work was carried out during the tenure of a John Simon Guggenheim Memorial Fellowship.

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XXIII. OXIDATION-REDUCTION POTENTIALS OF PNEUMOCOCCUS CULTURES.

II. EFFECT OF CATALASE.

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(*Received January 1st, 1931.*)

In previous communications the electrode potential behaviour of haemolytic streptococci [Hewitt, 1930, 1], *C. diphtheriae* [1930, 2], staphylococci [1930, 3] and pneumococci [1930, 4] has been described by the author and it has been shown that the oxidation-reduction potentials established in bacterial cultures depend upon the cultural conditions and upon the organism studied. The potentials developed in streptococcal cultures are roughly similar to those in pneumococcal cultures, but are quite different from those in staphylococcal and *C. diphtheriae* cultures.

Haemolytic streptococci and pneumococci form peroxide in aerated cultures, whilst staphylococci and *C. diphtheriae* do not, and it is becoming increasingly evident that peroxide formation is an important factor in bacterial behaviour. McLeod and Gordon [1923] classify bacteria on the basis of peroxide formation and account for the phenomenon of anaerobiosis on the grounds of the toxicity of peroxide. In his recent work on the effect of oxidation-reduction conditions on bacterial variation Todd [1930] finds that peroxide may have a selective inhibitory effect on a particular variant and thus peroxide formation may lead to selection of another variant less sensitive to peroxide.

In view of the importance of peroxide it is of interest to eliminate its effect and to determine what other differences exist between different bacteria. Addition of catalase to a peroxide-forming organism such as the pneumococcus should inhibit peroxide formation and thus reveal other characteristics in its oxidation-reduction behaviour.

In this paper are described the effects of catalase on the electrode potentials developed in pneumococcus cultures.

Methods.

The general methods and apparatus for the measurement of electrode potentials were the same as those previously described [1930, 1]. An inoculum of 0·1 cc. of a 24-hour broth culture of pneumococcus type II was made into 8 cc. of medium in each case and the potential was observed from the time

of inoculation, at frequent intervals for the first 12 hours and then less frequently. The incubator temperature was 37°.

Catalase preparations.

Liver catalase. The liver catalase preparation was made by the general method of Batelli and Stern [1904]. Fresh liver was minced finely, extracted with water, the cell debris was centrifuged down and the supernatant fluid was treated with 2 volumes of 98 % alcohol. The flocculent precipitate was filtered off, dissolved in water and reprecipitated with alcohol. The precipitate was filtered off on a Büchner funnel, drained thoroughly and dried *in vacuo* over sulphuric acid. The yield was approximately 1 g. of dried powder from 1 lb. of fresh liver, and the preparation showed marked catalase activity.

Bacterial catalase. The bacterial catalase, kindly provided by Dr E. W. Todd, was prepared as follows. *Micrococcus lysodeikticus*, grown on agar slopes for 48 hours, was suspended in saline and rendered sterile by incubation with chloroform, the chloroform was removed and the killed organisms were then centrifuged down, the supernatant fluid being discarded. The freshly centrifuged cells had only a weak catalase activity but when allowed to stand overnight the catalase activity had increased enormously. Possibly the intact cells react sluggishly with hydrogen peroxide and catalase escapes slowly either by diffusion or by lysis of the cells.

Blood catalase. Sterile, washed red blood corpuscles suspended in saline were used as a third source of catalase.

Results.

Liver catalase.

0·1 g. of the liver catalase preparation described above was dissolved in 10 cc. of water and filtered through a sterile Seitz filter. 1 cc. of this sterile 1 % solution was added to each 10 cc. of peptone infusion broth. Catalase broth was prepared by heating broth in a boiling water-bath for 30 minutes, cooling and adding the liver catalase, and inactivated catalase broth was prepared by adding the liver catalase and then heating the broth for 30 minutes. The catalase broth had marked catalase activity whilst the inactivated catalase broth, used in control experiments, had no catalase activity.

Aerobic cultures. In ordinary stationary aerobic cultures the potential fell to the same extent in the liver catalase broth and inactivated catalase broth. For the first 12 hours the catalase had no effect on the potential-time curve (Fig. 1). After the logarithmic phase of growth the potential of the culture in the inactivated catalase broth began to rise as is usual in pneumococcus cultures [Hewitt, 1930, 4] and the 48-hour culture had a potential 0·32 v. less negative than the 12-hour culture. In the catalase broth culture, on the other hand, the potential remained at the same low (*i.e.* reducing) level long after the logarithmic phase of growth and at the end of 50 hours had not

risen above the level of the 12-hour culture. The presence of catalase therefore prevents the disappearance of reducing conditions usually observed after the logarithmic phase of growth, although it has no effect on the initial establishment of reducing conditions. It seems evident that peroxide formation must be responsible for the disappearance of reducing conditions and the rise of potential since these are prevented by the presence of catalase. That peroxide formation is effective at such low (reducing) levels of potential is somewhat surprising and will be commented upon later.

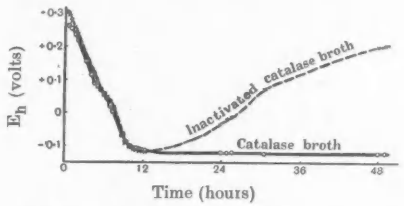

Fig. 1. Aerobic peptone infusion broth cultures of pneumococcus (liver catalase).

Aerated cultures. It was shown in a previous paper [1930, 4] that when cultures of pneumococci were subjected to vigorous aeration, the potential fell only to a slight degree and then rapidly rose to the high level of potential which corresponds with the formation of detectable amounts of peroxide. It was found that inactivated liver catalase had no effect on the electrode potentials of aerated pneumococcus cultures. In Fig. 2 the upper curve

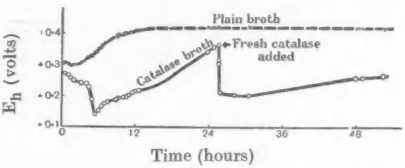

Fig. 2. Aerated pneumococcus cultures (liver catalase).

represents an aerated broth culture of pneumococci, and a similar culture containing inactivated catalase gave an almost indistinguishable curve. The potential fell only 0·007 v. then rose rapidly, and in 10 hours reached $E_h = +0{\cdot}4$ v. at which level peroxide may be detected chemically in the culture. It was found, however, that in a similar culture containing active liver catalase the potential fell 0·13 v. and then commenced to rise. After 25·5 hours the potential had reached $E_h = +0{\cdot}36$ v. At this stage a further quantity of liver catalase was added (equal in amount to that originally added). This resulted in an immediate rapid drop in potential amounting to 0·15 v. in 40 minutes. This effect was evidently due to decomposition of the peroxide present by the fresh catalase added, the original catalase having been destroyed by the continuous production of peroxide by the pneumococci.

A further interesting observation was made with regard to the effect of catalase. After 53 hours' incubation the aerated broth culture, the aerated inactivated catalase broth culture and the aerated catalase broth culture, were subcultured on blood agar. After 18 hours' incubation there was no growth from the two former inoculations but copious growth from the catalase broth culture. Peroxide formation had sterilised the broth cultures but the presence of catalase had protected the catalase broth culture. It is worthy of note that the aerated catalase broth culture was markedly turbid after 53 hours' growth showing that pneumococci flourish when the oxygen supply is abundant, provided that the harmful effects of peroxide are removed. This confirms the view previously expressed [1930, 1, 2, 3] that many bacteria grow most luxuriantly in the presence of a generous oxygen supply. This effect may be obscured with organisms which do not contain catalase owing to the toxic effect of the peroxide which may be formed when the oxygen supply is abundant.

Bacterial catalase.

0·05 cc. of a sterile killed preparation of *M. lysodeikticus*, centrifuged down to a thick suspension, was added to each 10 cc. of broth. For bacterial catalase broth the broth was heated for 30 minutes in a boiling water-bath and cooled before the addition of the preparation whilst for inactivated bacterial catalase broth, used as a control, the preparation was added to the broth before heating.

Aerobic cultures. In ordinary stationary aerobic cultures the effect of bacterial catalase was similar to that observed with liver catalase (Fig. 3).

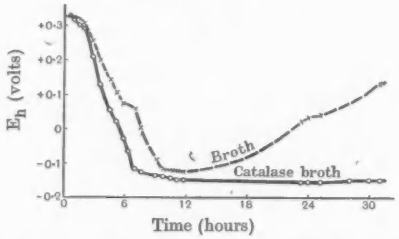

Fig. 3. Aerobic pneumococcus cultures (bacterial catalase).

After 30 hours' incubation the culture containing bacterial catalase had the same low potential reached after 12 hours' growth whereas in a similar culture not containing catalase the potential had risen 0·16 v. It may be worthy of note that whereas the boiled inactivated suspension of *M. lysodeikticus* had no effect on the establishment of reducing conditions, the unheated but killed bacteria appeared to facilitate reduction, since the potential fell rather more rapidly and to a slightly lower level in the bacterial catalase broth. This effect was not seen with liver catalase nor with blood catalase and may possibly be due to certain enzymes or accessory substances in the killed bacteria. The

point of inflection on the plain broth curve just below $E_h = + 0{\cdot}1$ v. has been observed on a number of occasions but its significance is uncertain.

Aerated cultures. Bacterial catalase had the same effect as liver catalase on the electrode potential behaviour of vigorously aerated cultures of pneumococci (Fig. 4). In the aerated broth and the aerated inactivated bacterial

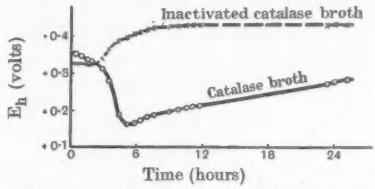

Fig. 4. Aerated cultures (bacterial catalase).

catalase broth cultures the potential fell only 0·004 to 0·007 v. and then rose fairly rapidly reaching the peroxide level ($E_h = + 0{\cdot}4$ v.) after 6 hours' incubation. In the bacterial catalase broth, however, the potential fell to the extent of 0·19 v. and then rose very slowly and no peroxide was formed even after 30 hours' incubation.

Blood catalase.

Blood catalase broth was prepared by adding 0·1 cc. of sterile washed red blood corpuscles to each 10 cc. of broth.

Stationary aerobic cultures. In stationary aerobic cultures containing blood catalase the potential fell in the usual way except that a flat portion of the curve occurred at a level of $E_h = + 0{\cdot}3$ v. (Fig. 5). The form of the curve suggested that some poising oxidation-reduction system had to be reduced before the potential could fall. It seems probable that some haemoglobin system may be responsible for this effect. In this connection it may be mentioned that only reversible oxidation-reduction systems can give a well-defined

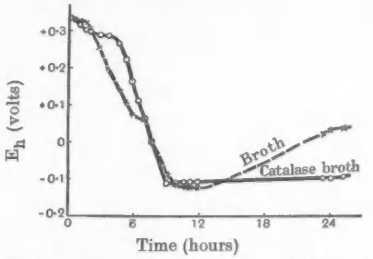

Fig. 5. Aerobic pneumococcus cultures (blood catalase).

potential at an unattackable electrode, but other systems may affect the potential-time curve of a culture and have a poising effect, since other reducing actions (which would be reflected in the fall in potential) cannot occur until the poising system is itself reduced.

The potential in a stationary aerobic blood catalase broth culture of pneumococci remained at a low level after 30 hours' incubation, whereas the potential in a plain broth culture had risen in the usual way. Blood catalase thus had the same effect in maintaining reducing conditions after the cessation of active proliferation as had liver and bacterial catalase.

Aerated cultures. The flat portion of the curve which was observed in stationary cultures was noticed also in vigorously aerated blood catalase broth cultures (Fig. 6). In the presence of blood catalase the potential of aerated

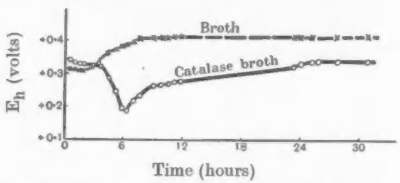

Fig. 6. Aerated pneumococcus cultures (blood catalase).

cultures fell to a much greater extent and rose more slowly than in plain broth cultures. Thus blood catalase had the same general effect on the potential as had liver and bacterial catalase preparations.

Discussion.

The presence of catalase has very marked effects on the oxidation-reduction conditions of pneumococcus cultures. By using catalase from different sources—from liver, bacteria and blood—it is possible to eliminate the interfering effects inherent in the use of enzymes which cannot be isolated in the pure state but which are always contaminated with other substances.

In ordinary aerobic pneumococcus cultures the presence of catalase appears to have no effect on the establishment of reducing conditions during the phase of active proliferation, but after this phase the electrode potential in a plain broth culture commences to rise, whilst in the presence of active catalase the potential remains at a low level for a considerable time. It seems evident therefore that the usual rise in potential, after the logarithmic phase of growth of pneumococci, is due to peroxide formation, which evidently occurs long before peroxide can be detected chemically. The hydrogen peroxide formed in oxidation-reduction processes is, therefore, immediately decomposed by catalase, before it has time to affect the electrode potential, which remains at a low (*i.e.* highly reducing) level. The electrode potential behaviour of an aerobic pneumococcus culture containing catalase is therefore roughly similar to that of cultures of staphylococcus or *C. diphtheriae* which produce their own catalase.

In vigorously aerated pneumococcus cultures proliferation occurs very slightly, if at all, and the potential shows only a slight tendency to fall (0·004 to 0·007 v.) and then rapidly rises to the level at which peroxide may be

detected chemically. When catalase is present, however, the potential falls appreciably (0·13 to 0·19 v.) and rises only slowly. As long as active catalase is present the level corresponding to peroxide accumulation is not reached but the catalase present is gradually destroyed by the active oxidising system. When fresh catalase was added to an aerated culture from which the catalase had disappeared there was an immediate rapid drop in potential.

50-hour aerated broth cultures of pneumococci are sterile but in aerated cultures containing catalase the bacteria still proliferate actively when sub-cultured after 50 hours' incubation. Growth is negligible in aerated plain broth cultures of pneumococci but in aerated catalase broth cultures growth is very good, showing that pneumococci flourish when the oxygen supply is abundant provided that the toxic effect of peroxide is eliminated.

Although there is a considerable fall in potential (*circa* 0·15 v.) in aerated catalase broth cultures of pneumococci, this fall is much less than that observed with staphylococci and *C. diphtheriae* (0·35 to 0·45 v.). This suggests that there are differences in reducing power of different organisms quite apart from the catalase- and peroxide-forming functions. It is by no means certain, therefore, that the failure of obligate anaerobes to grow aerobically is due solely to potential peroxide production in the absence of catalase. Not only the toxic effect of peroxide but also inability to effect oxidation-reduction processes at high levels of electrode potential may account for the phenomena of anaerobiosis.

Bacterial peroxide and hydrogen peroxide have a number of properties in common.

(1) They give the same chemical reactions (*e.g.* the benzidine-peroxidase reaction);

(2) display similar electrode-potential behaviour [Hewitt, 1930, 1];

(3) are both decomposed in the presence of catalase.

These facts suggest that bacterial peroxide and hydrogen peroxide if not identical must be very closely related compounds, and my experiments, so far, have failed to reveal any differences between them.

Apart from their catalase effect *M. lysodeikticus* and red blood corpuscles have other effects on the oxidation-reduction conditions developed in pneumococcus cultures. Killed *M. lysodeikticus* appeared to accelerate reduction effects. This reducing effect was destroyed by heating and may be due to the presence of reducing enzymes or accessory substances. Red blood corpuscles, on the other hand, exerted a poising effect possibly due to some haemoglobin system which itself had to be reduced before further reducing effects could be developed in the culture.

SUMMARY.

1. Some of the characteristic differences in behaviour between pneumococci and catalase-forming bacteria disappear when catalase preparations are added to the pneumococcus culture.

2. When catalase has been added the electrode potential of aerobic pneumococcus cultures remains at a low level long after the logarithmic phase of growth.

3. In aerated cultures the potential falls to a much lower level and growth is much more luxuriant when catalase has been added.

4. Bacteria differ in oxidation-reduction behaviour in respects other than that of peroxide formation.

5. Bacterial peroxide and hydrogen peroxide possess many properties in common.

6. Indications were obtained of an acceleration of reduction effects by killed aerobic bacteria (*M. lysodeikticus*) and of a poising effect by erythrocytes.

The author is indebted to Dr R. G. White and Dr E. W. Todd for their continued help and encouragement.

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XXIV. THE TEMPERATURE COEFFICIENTS AND ENERGY EXCHANGES OF THE CITRIC ACID DEHYDROGENASE OF CUCUMBER SEEDS.

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From the Biochemical Laboratory, Cambridge.

(*Received January 1st, 1931.*)

Among modern theories of chemical reaction the activation theory has proved to be one of the most fruitful not only in the hands of the physical chemists who were primarily responsible for its development, but also as a tool for the biochemist in his attempt to elucidate the mechanism of enzyme action. The theory postulates that an exceptionally high energy content is the most important of the criteria which determine the activated state preceding reaction, because the change of reaction velocity with changing temperature follows an expression of the same form as the law which expresses the variation with temperature of the number of molecules whose energy content exceeds an assigned value. The acceptance of this postulate has led to the view that in general the energy content of a molecule must be increased by an energy increment (the "critical increment" or "energy of activation") as a necessary prelude to chemical change of that molecule.

In terms of this theory the function of a catalyst is to aid the molecules of the reactant to attain the activated state—that is, to lower the critical increment. It is the examination of the way in which a heterogeneous catalyst lowers the critical increment of the molecules of a reactant which is germane to the problem of the mode of action of an enzyme. The action of the catalyst here can be described rather loosely as the straining of the substrate molecule, and this description has the merit of suggesting that the catalyst does work upon or yields energy to the molecule in activating it; but since it has become widely recognised that the forces in play at the boundary of any two phases are not purely physical but are chemical forces of valency, a more helpful picture of catalysis results from the legitimate postulate that the catalyst and reacting substance combine chemically, and that in the process the molecules of the reactant become activated.

This hypothesis may be applied to enzyme reactions in two ways. In the first method the measure of the critical increment may be ignored and attention may be concentrated on the changes wrought in the substrate molecule during activation, *i.e.* upon the straining of the molecule, which is most easily pictured as isomerisation. This mode of attack was employed by Quastel [1926]

and Quastel and Wooldridge [1927 and 1928] in studies of the enzyme systems of resting *B. coli*, although it is evident that Quastel did not regard the union of enzyme and substrate as a simple chemical reaction. The second method is to ignore the qualitative effect of activation on the substrate molecule and to examine the energy exchanges between the enzyme, the substrate and the surrounding system. This quantitative method has its own limitations—for instance it cannot lead us to a reason why citric acid dehydrogenase oxidises citric acid to acetonedicarboxylic acid while, in the absence of the dehydrogenase, reduction of the critical increment of citric acid by raising the temperature causes it to pass into aconitic acid—but it yields valuable data in other directions. The energy changes are measured indirectly by calculation from observations of the kinetics of the enzyme action, a fact of importance because at present the kinetics alone among the characteristic properties of an enzyme are amenable to exact quantitative measurement.

A rational description of the energy relations accompanying an enzymic reaction can be given on the assumption that the union of enzyme and substrate is a simple chemical reaction, following the hypothesis developed by Michaelis and Menten [1913] to account for the observed facts relating to the effect of substrate concentration on the rate of sucrose hydrolysis by saccharase, and subsequently applied with such marked success to the elucidation of many problems in the kinetics of this and of other enzymes. Their theoretical deduction of the relation between substrate concentration and initial reaction velocity for saccharase may be generaused in the following way. Let E represent the molecule of the enzyme, S the molecule of the substrate, and P the molecule of the product (or of one of the products if there are several). Then the enzyme and substrate are assumed to combine reversibly to form the compound ES which breaks down irreversibly into $E + P$, according to the equation $E + S \rightleftarrows ES \rightarrow E + P$. At the commencement of reaction, the total molar concentrations of enzyme, substrate and the compound ES being represented by e, s and p respectively, the dissociation constant of the compound ES is given by

$$K_m = \frac{(s-p)(e-p)}{p} = \frac{s(e-p)}{p}$$

to a close approximation because s is supposed to be much greater than e; and

$$p = \frac{es}{K_m + s}.$$

If the velocity constant of breakdown of ES to $E + P$ is written k, then the actual initial velocity of reaction

$$V = kp = \frac{kes}{K_m + s}.$$

This expression foretells the shape of curve obtained by plotting the initial velocity of reaction against substrate concentration to be a rectangular hyperbola, since V increases rapidly at first as s is increased from zero and then when s exceeds K_m further increase causes V to approach asymptotically the

limiting value $V_{max.} = ke$. Further, V will reach a figure equal to half the limiting value when $s = K_m$. Thus for an enzyme which follows the Michaelis equations, measurements of the initial rate of the reaction with varying substrate concentrations render it possible to calculate the value of the Michaelis constant K_m, which is one of the most characteristic constants of an enzyme. The assumption that the velocities of formation and dissociation of the enzyme-substrate compound are much larger than the velocity of its breakdown to the products of reaction is implicit in the above derivation; but there is evidence which suggests its validity [*cf.* Haldane, 1930, p. 41], and for the present it will be accepted as correct.

In considering the energetics of an enzyme action represented as above, there are three important and characteristic quantities which must be carefully distinguished. These are (1) the heat of formation of the compound ES, (2) the energy of activation, otherwise the critical increment, of the compound ES, which is necessary to enable it to react to yield the product P; and (3) the heat of the reaction $S \rightarrow P$, which is the total heat of reaction of the complete process, and which is not of necessity related to the other heat quantities. The first quantity can be calculated when the equilibrium constant of the reaction $E + S \rightleftarrows ES$ is known at two or more temperatures, with the aid of van't Hoff's reaction isochore

$$\frac{d(\log_e K)}{dT} = -\frac{Q}{RT^2},$$

where K is the equilibrium constant of the reaction, Q the heat of formation, T the absolute temperature, and R the gas constant. When an enzyme follows the Michaelis laws and the value of the Michaelis constant can be measured at different temperatures it is therefore possible to determine the heat of formation of the enzyme-substrate compound. The heat of activation of this compound can be calculated by the use of a similar expression due to Arrhenius, when the velocity constant k of its breakdown can be measured at two temperatures; and finally the heat of the catalysed reaction can be calculated by use of the isochore if the change of the total equilibrium with change of temperature is known. As the present considerations deal only with the initial stages of reaction this heat quantity is left out of account because it cannot be calculated from observations of the early stages of the reaction; attention has therefore been confined to the first two quantities and the relation between them.

The calculation of the first two of the separate heat quantities detailed above depends on the measurement of the temperature coefficients of the Michaelis constant and of the velocity constant k respectively, and since Haldane [1930, p. 38] has suggested that the characteristically low Michaelis constants of oxidising enzymes as compared with those of hydrolytic enzymes may indicate a fundamental difference between the two types, it is of immediate interest to discover whether the temperature coefficient of K_m is of the

same magnitude for enzymes of the two types[1]. Before any generalisations can be made it will be necessary to obtain data about many enzymes, but as accurate data for the hydrolysing enzyme saccharase are already on record any figures for an oxidising enzyme will be of value for comparison. To obtain suitable data demands observations upon some typical oxidising enzyme, the desiderata which govern the choice being that the Michaelis constant is small and is susceptible of reasonably accurate measurement over some suitable temperature range.

Such an enzyme is the citric acid dehydrogenase described recently by Thunberg [1929]. Fig. 1 embodies data given by Thunberg in his paper, and

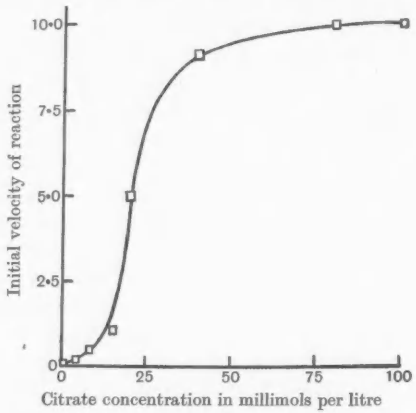

Fig. 1. The relation of initial velocity of reaction to substrate concentration for the citric acid dehydrogenase of cucumber seeds. Drawn from data given by Thunberg [1929].

it shows that the dehydrogenase has a low Michaelis constant (which can be read approximately from the graph as the substrate concentration at which the initial velocity reaches half its maximum value) and suggests that it should be possible to measure this constant with considerable accuracy. The curve deviates from the ideal rectangular hyperbola to a considerable extent at the lowest substrate concentrations because it is impossible with the technique available to measure at all accurately the initial rate of reaction with very small concentrations of substrate. The experimental method consists of the observation of the time taken for a standard small amount of the substrate to be oxidised, the concentration of the substrate at the commencement of the observation being known. It is thus implicitly assumed that the velocity throughout the observation remains constant at the initial value, and error is introduced because this can only be true with relatively high concentrations. Fortunately this difficulty vanishes if the lowest concentration at which

[1] Professor J. B. S. Haldane suggested this problem to the author, pointing out the importance which would be attached to the discovery of a high temperature coefficient of K_m for an oxidising enzyme.

accurate measurement of the reaction time is possible is less than the concentration at which the enzyme is half saturated by its substrate, because corrections can then be applied in order to calculate the true initial velocity from the apparent initial velocity of the reaction.

These considerations led the writer to attempt the measurement of the temperature coefficients of K_m and of the velocity of reaction for citric acid dehydrogenase in order to gain an insight into the energy exchanges occurring during its action.

Experimental methods.

All observations were made by the method of Thunberg [1928]. The dehydrogenase was allowed to act on its substrate in a Thunberg vacuum tube, and the hydrogen acceptor used was methylene blue. Each individual observation consisted in noting the time taken for the reduction of a standard amount of methylene blue under carefully controlled conditions of temperature, p_H, substrate concentration, enzyme concentration, etc.

The enzyme solutions employed in this work were prepared from seeds of the cucumber (*Cucumis sativa*) which were obtained from Messrs Weibull of Landskrona, Sweden, and bore the trade name "Västeras Grön Druv." For each series of observations a sufficient quantity of the seeds was shelled to give 5 g. of kernels, which were then ground in a mortar with 25 cc. of 0·87 % dipotassium hydrogen phosphate for 20 minutes. The milky liquid so obtained was centrifuged for 10 minutes at a speed of approximately 4000 r.p.m., at the end of which time it had separated into three layers. The bottom layer contained solid residue, the second layer which was still rather milky in appearance contained the active material, and the top layer consisted of the seed fats. The aqueous layer was separated from the others and was ready for immediate use.

In a single experiment a series of Thunberg vacuum tubes was prepared each containing 0·4 cc. of a solution of potassium citrate of known concentration. To each tube in turn were added 0·3 cc. of a 1 in 60,000 solution of methylene blue and 0·5 cc. of the enzyme solution; the tubes were then evacuated at the water-pump for 90 seconds, closed and immersed in a water-bath of which the temperature was kept constant within 0·05°. The concentrations of the potassium citrate solutions added at first were so graded that the complete reaction mixtures formed a series in which the citrate concentration varied from 4 *mM* to 400 *mM*, and other factors were constant. The time for the complete decoloration of the methylene blue in each tube was observed.

This method is similar in essentials to the one employed by Thunberg [1929] for the estimation of citric acid by means of this dehydrogenase, and the following comments may be made on it.

(1) As the total volume of reaction mixture in each tube is only 1·2 cc., it is essential that the tubes be thoroughly dried before use.

(2) The reaction is accelerated by fats and oils, so that the reaction mixture must not under any circumstances be allowed to come into contact with the grease lubricating the tap at the top of the tube. It is an advantage to put the whole of the enzyme preparation in a small Büchner flask and evacuate the vessel for a few minutes before making up the reaction mixtures, as the amount of frothing in the individual vacuum tubes during evacuation will be greatly diminished thereby. For the same reason the vacuum tube must be completely immersed in the thermostat. If the top of the tube is above the water level of the bath, distillation will occur within it and water from the reaction mixture will condense within the cooler tap and come into contact with the tap grease as it runs down the tube into the reaction mixture again. The reduction time of the methylene blue may be decreased by 50 % if this precaution is not taken.

(3) Evacuation by a good water-pump for 90 seconds reduces the oxygen concentration in the tube sufficiently.

(4) A fresh enzyme solution must be prepared for each series of observations, and kept at 0° if not used immediately.

(5) The measurements must be made at the optimum p_H of the system [*cf.* Nelson and Bloomfield, 1924].

(6) It was found that the results obtained always contained some irregular variations which could not be eliminated, therefore in the determination of the temperature coefficients the writer, after becoming conversant with the technique of the observations, performed a series of five experiments at 25° with different samples of active material, and from the readings calculated the mean value of K_m at that temperature. In the same way a mean value of K_m at 35° was measured and the temperature coefficient of the constant was determined from the two mean values.

Experimental results.

Using the enzyme in phosphate solution as described above, it was found that at the end of the period of observation the p_H of the reaction mixture as determined by means of the quinhydrone electrode was always $7{\cdot}84 \pm 0{\cdot}03$. On account of the necessity of measuring the temperature coefficient at the optimum p_H of the system, a preliminary examination of the p_H-activity relationship of the dehydrogenase was made in which the initial velocity of reaction was determined at different hydrogen ion concentrations, all measurements being made with substrate concentrations high enough to ensure that the enzyme was fully saturated by its substrate. The activity was found to rise from a minimum in acid solutions (the enzyme was precipitated from solution below p_H 3·5) to a maximum at about p_H 7, and in more alkaline solutions the activity remained constant until a p_H above 10 was reached. This result is expressed in the curve shown in Fig. 2. All experiments carried out with the enzyme in dilute secondary phosphate solution were therefore done within the optimum activity range of the system.

The reduction time of the methylene blue in each of the tubes of all ten experiments is given in Table I. To calculate the value of K_m from the figures for each experiment it is first necessary to work out the initial velocity of reaction V at each concentration of citrate, relative to the initial velocity $V_{max.}$ which is attained when the citrate is sufficiently concentrated to saturate the

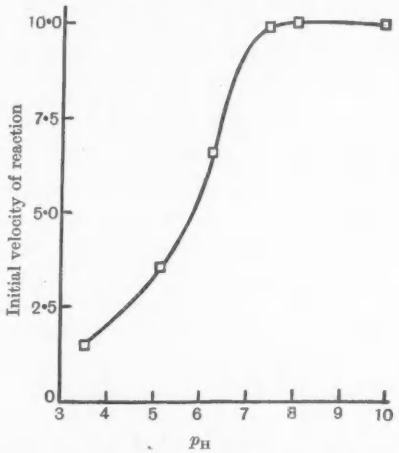

Fig. 2. The p_H-activity curve for the citric acid dehydrogenase.

enzyme fully. The velocity of reduction of the methylene blue in each tube is proportional to the reciprocal of the reduction time, therefore the reciprocal of each of the reduction times recorded in each experiment has been tabulated and mutiplied by 100 for convenience. The velocity of oxidation of the citrate in any tube is taken as proportional to the difference between the velocity of reduction of methylene blue in that tube and the velocity of reduction of methylene blue in the blank tube of the experiment (which contains no citrate). Finally, as in each experiment it was found that the initial velocity reached a maximum when the citrate concentration was 200 mM, the initial velocity at this concentration is taken as unity and the initial velocity at each of the other concentrations is expressed as a fraction of the initial velocity with 200 mM citrate, under the title $\frac{V}{V_{max.}}$. The successive figures obtained in this way from Exp. 1 are set out in Table II as an example of the procedure adopted to calculate values of $\frac{V}{V_{max.}}$ at each concentration in each experiment. In Table III the whole of the figures for $\frac{V}{V_{max.}}$ obtained in the complete series are collected together.

To find the heat of activation of the enzyme-substrate compound it was necessary to measure the reaction velocity with citrate concentrations large enough to saturate the enzyme completely; readings were taken both at 25°

Table I.

Reduction of methylene blue by citric acid in presence of citric acid dehydrogenase at p_H 7·84. Each tube contained 0·5 cc. of enzyme solution in 0·87 % secondary potassium phosphate, 0·4 cc. of potassium citrate solution of varying concentrations, and 0·3 cc. of a 1 in 60,000 solution of methylene blue.

Exp. No.	Temp.	Reduction time in minutes									
1	25°	120	115	92	92	100	63	32	27	23	27
2	,,	71	70	54	64	64	54	30	27	21	23
3	,,	56	70	44	64	47	41	*	21	21	24
4	,,	76	85	76	81	83	59	43	38	37	31
5	,,	99	93	98	92	89	69	75	37	33	30
6	35°	61	60	60	53	58	41	24	26	26	24
7	,,	52	55	50	43	41	36	22	22	23	22
8	,,	64	64	44	*	54	35	22	19	19	19
9	,,	76	72	64	66	54	49	26	24	22	22
10	,,	71	65	55	57	59	46	31	29	27	26
Citrate *mM*		0	4	8	16	20	40	80	100	200	400

* Indicates no figure obtained.

Table II.

Calculation of $\frac{V}{V_{max.}}$ at each concentration for Exp. 1.

Citrate *mM*	0	4	8	16	20	40	80	100	200
Reduction time minutes	120	115	92	92	100	63	32	27	23
$\frac{100}{\text{Reduction time}}$	0·83	0·87	1·09	1·09	1·0	1·58	3·11	3·7	4·35
$V = \frac{100}{\text{Reduction time}} - 0{\cdot}83$	0	0·04	0·26	0·26	0·17	0·75	2·28	2·87	3·51
$\frac{V}{V_{max.}}$	0	0·01	0·07	0·07	0·05	0·21	0·64	0·82	1·0

Table III.

Values of $\frac{V}{V_{max.}}$ for all tubes of Exps. 1 to 10, calculated from the reduction times given in Table I.

Exp. No.	$\frac{V}{V_{max.}}$								
1	0	0·01	0·07	0·07	0·05	0·21	0·64	0·82	1
2	0	0	0·12	0·04	0·04	0·12	0·55	0·65	1
3	0	0·12	0·16	0·08	0·11	0·22	—	1	1
4	0	0·1	0	0·05	0·06	0·23	0·74	0·98	1
5	0	0·03	0	0·04	0·06	0·22	0·57	0·81	1
6	0	0·01	0·01	0·11	0·04	0·37	1·15	1	1
7	0	0·04	0·03	0·16	0·2	0·35	1·08	1·08	1
8	0	0	0·2	—	0·07	0·35	0·81	1	1
9	0	0·03	0·09	0·07	0·18	0·25	0·86	0·97	1
10	0	0·06	0·18	0·15	0·13	0·33	0·80	0·89	1
Citrate *mM*	0	4	8	16	20	40	80	100	200

and at 35° with the same enzyme preparation. These measurements were made on tubes which contained 0·5 cc. of enzyme solution, 0·3 cc. of 1 in 60,000 methylene blue and 0·4 cc. of a solution of potassium citrate, which made the citrate concentration of the reaction mixture 400 *mM*. The ratio $k_{35°}/k_{25°}$ was found to be 1·61. Repetitions of the observation with other enzyme preparations gave values of 1·70 and 1·64; and one observation over

the wider temperature range from 15° to 35° gave the figure 2·71 for the ratio of the maximum velocities of reaction at these two temperatures.

In addition to the measurement of the temperature coefficients, some observations on other qualitative and quantitative properties which have not yet been fully investigated may be reported briefly. The fatty layer which separates on centrifuging the cucumber seeds ground up in phosphate possesses the power of accelerating the action of the enzyme: a fact which has also been noted by Thunberg (private communication to the author). Citraconic acid accelerates the action slightly, but on the other hand aconitic acid inhibits competitively, just as was found for the citric acid dehydrogenase of liver by Bernheim [1928]. Finally it was noticed that under certain conditions the enzyme preparation is much more slowly destroyed on standing than is suggested by Thunberg [1929] and other workers of his school, *e.g.* Scherstén [1929]. This was investigated in order to ensure that the destruction of the enzyme at 35° was not so great as to cause serious error in the measurement of the temperature coefficients. In a typical experiment it was found that under the standard conditions specified above, 0·5 cc. of a freshly prepared enzyme solution caused the reduction of 0·4 cc. of 1 in 60,000 methylene blue in presence of 400 *mM* citrate in 49 minutes, while after the enzyme had been kept at 35° for one hour it brought about an identical reduction in 54 minutes. The destruction of the enzyme was therefore slight. As it is to be expected that the destruction in presence of the substrate occurs more slowly, the error so introduced into the measurement of the temperature coefficients is negligible.

Calculation of the energy exchanges.

(1) *The heat of formation of the enzyme-substrate compound.* The first step is to calculate the value of K_m at 25° and at 35°. From the data of Table III it can be seen that the figures for $\frac{V}{V_{\max.}}$ are extremely scattered for concentrations less than 40 *mM* and cannot be used in the determination of the temperature coefficient. This indeed is to be expected because the concentration of methylene blue in the reaction mixtures is approximately 10 *mM* (reckoning the molecular weight as 400), and serious errors are introduced when the concentration of the methylene blue approaches that of the citrate. The figures for $\frac{V}{V_{\max.}}$ are however definitely greater at 35° than at 25° for 40 *mM* and 80 *mM* citrate solutions; and the figures for 100 *mM* solutions show plainly that the enzyme is saturated by its substrate at lower concentrations of citrate at 35° than at 25°. The form of the calculation by which K_m is derived from the figures for $\frac{V}{V_{\max.}}$ magnifies greatly the experimental errors if $\frac{V}{V_{\max.}}$ is greater than 0·5, therefore the calculations which follow are based on the $\frac{V}{V_{\max.}}$ figures for the 40 *mM* citrate solutions in each experiment.

Since at a substrate concentration s,

$$V = \frac{kes}{K_m + s},$$

and since the limiting value $V_{max.} = ke$, it follows that

$$\frac{V}{V_{max.}} = \frac{s}{K_m + s},$$

and therefore

$$K_m = s\left(\frac{V_{max.}}{V} - 1\right).$$

From each experiment of the series K_m has been calculated by means of this formula, and the figures are given in Table IV.

Table IV.

The Michaelis constants calculated from the data of Table III.

Exp. No.	1	2	3	4	5	6	7	8	9	10
K_m	150	293	142	134	142	68	74	74	120	81

Although these results are scattered, there is no overlapping of the figures for the first five experiments at 25° with those for the last five at 35°, and it is clear that there is a considerable shift of K_m due to a temperature change of 10°. The mean value at 25° is 172 and at 35° it is 83; a decrease which corresponds to a notable heat of reaction.

Before accepting these as the best available figures the errors they involve must be discussed. First there are the random errors of measurement which are partially eliminated by taking the mean values at each temperature and are probably quite small. Secondly, errors are introduced by a real difference between the Michaelis constants of different enzyme preparations. The figures for Exps. 2 and 9 vary from the means for their respective groups so much as to decide this point. Thirdly, in each figure is incorporated an error due to the relatively large concentration of methylene blue which it is necessary to use in the reaction mixture. The first and second errors are not serious because they affect the figures for K_m at both temperatures to the same extent, and will not therefore alter the ratio $K_m^{35°}/K_m^{25°}$ significantly. On the other hand, the third error varies in magnitude as the Michaelis constant changes, and as the change of Michaelis constant with temperature here recorded is much greater than has heretofore been discovered, it is necessary to show that the change cannot be due to this error distorting the results. The treatment which follows here shows that the error introduced is quite small, and although the true values of K_m are all rather smaller than those given in Table IV the ratio of the mean value at 35° to the mean value at 25° is almost unaffected.

The error is due to the fact that the concentration of the methylene blue is so large that it causes the citrate concentration to fall by an appreciable amount during the reaction. If t_0 be the reduction time of the blank tube and t_{40} the reduction time of the tube containing 40 mM citrate in any experiment, then in the tube containing the citrate the number of millimols of citrate reduced by the "blank process" is about $10 \times t_{40}/t_0$, since the initial concentration of methylene blue is approximately 10 mM. The number of millimols reduced by the citrate is therefore $10\,(1 - t_{40}/t_0)$, and the concentration of the citrate must fall by this amount during the reaction. Thus for experiment 1 this

quantity is $10\,(1 - 63/120)$, or 4·75 *mM*. In calculating K_m it is necessary therefore to take into account that the citrate concentration fell from 40 to 35·25 *mM*, and this can be done with sufficient accuracy by substituting for the initial concentration the average concentration during the experiment, *i.e* 37·6 instead of 40. Then the corrected value of K_m is $37{\cdot}6\left(\frac{V_{\max.}}{V} - 1\right)$ and this is $37{\cdot}6\left(\frac{1}{0{\cdot}21} - 1\right)$, or 141, instead of 150, which is the uncorrected figure. In Table V the corrected Michaelis constants for each experiment are tabulated.

Table V.

The figures for the Michaelis constant corrected for the fall of citrate concentration during the reaction.

Exp. No.	1	2	3	4	5	6	7	8	9	10
K_m (corr.)	142	281	137	130	137	65	71	70	115	78

Mean value K_m at 25° 165 *mM*.
" " " 35° 80 "

From these figures the heat of formation of the enzyme-substrate compound is calculated by substitution in the integrated form of the reaction isochore. The expression

$$\frac{d(\log_e K)}{dT} = -\frac{Q}{RT^2}$$

can be integrated on the assumption that Q is constant, giving

$$\log_e \frac{K_2}{K_1} = \frac{Q(T_2 - T_1)}{RT_1T_2},$$

where K_2 and K_1 are the equilibrium constants at T_2 and T_1 respectively. The heat of reaction is generally assumed to be constant over a small temperature range such as 10°, so by substitution, since the equilibrium constant of the forward reaction $E + S \rightarrow ES$ is the reciprocal of the Michaelis constant:

$$2{\cdot}3 \log_{10} \frac{K_m^{25°}}{K_m^{35°}} = \frac{Q}{1{\cdot}99} \times \frac{(308 - 298)}{298 \times 308},$$

therefore

$$2{\cdot}3 \log \left(\frac{165}{80}\right) = \frac{Q}{1{\cdot}99} \times \frac{10}{298 \times 308}$$

and

$$Q = +\,2{\cdot}3 \times 0{\cdot}3144 \times 1{\cdot}99 \times \frac{298 \times 308}{10} = +\,13460 \text{ cal.}$$

The heat liberated by the combination of citric acid dehydrogenase and citric acid is 13460 cal. per g. molecule of citric acid.

(2) *The heat of activation of the enzyme-substrate compound.* The mean of the three figures obtained for the ratio of maximum reaction velocity at 35° to the maximum velocity at 25° is 1·65, and by substitution in the expression

$$E = \frac{RT_1T_2}{T_2 - T_1} \log_e \frac{k_2}{k_1},$$

the energy of activation is found to be 9139 cal. per g. molecule.

Discussion.

The observations described above lead definitely to the conclusion that when the citric acid dehydrogenase combines with its substrate a large amount of heat is set free, and although the figure given is not accurate, it can be said with confidence that the heat of formation of the enzyme-substrate compound is about 12,000 or 15,000 cal. per g. molecule. This is a fairly large figure, and larger indeed than the heat of activation of the enzyme-substrate compound, which is about 9000 cal. per g. molecule. As far as the writer is aware the only other enzyme for which both the corresponding energy values have been determined is yeast saccharase. Euler and Laurin [1920] measured the heat of formation of the saccharase-sucrose compound and concluded that it was about 2000 cal. per g. molecule. On the other hand, Nelson and Bloomfield [1924] in the course of a very careful study of the kinetics of this enzyme found that the heat of formation of the enzyme-substrate compound was zero within the limits of experimental error. The heat of activation of the saccharase-sucrose compound was found by them to be 8400 cal. per g. molecule at 30°, in agreement with the figures obtained by Euler and Laurin and other workers.

It is evident that although the energy of activation of the enzyme-substrate compound is of the same order for yeast saccharase and for citric acid dehydrogenase at 30°, the heat of formation of the compound at the same temperature is quite large for the dehydrogenase and zero for the saccharase. Whether this distinction is a general one between oxidising and hydrolysing enzymes cannot be decided until similar data are available for many other enzymes of both classes, but the possibility must be born in mind. If it holds, it will take its place with the generalisations that hydrolyses proceed with much smaller heats of reaction than oxidations, and that the Michaelis constants of oxidising enzymes are much smaller than those of hydrolysing enzymes.

The fact that in the case of the citric acid dehydrogenase the heat of formation of the enzyme-substrate compound is greater than the energy of activation of the same compound, suggests that the action of the enzyme may be pictured in terms of the energy exchanges as follows: the enzyme molecule unites with a molecule of substrate and heat energy is thereby released which can immediately be absorbed by the enzyme-substrate compound. This absorption of energy by the compound serves to activate it and it then passes from the activated state to the final state of enzyme molecule + molecules of the product or products. Such a sequence of energy exchanges could not however apply when an enzyme-substrate compound has a heat of formation less than its energy of activation, as is the case with saccharase.

Summary.

The Michaelis constant of the citric acid dehydrogenase of cucumber seeds has been measured at 25° and at 35°. At 25° it is $1{\cdot}65 \times 10^{-4}$ M, and at 35° it is 8×10^{-5} M. Thus the temperature coefficient $Q_{10^\circ} = 0{\cdot}48$ at 30° and

the heat of formation of the citric dehydrogenase-citric acid compound as calculated from the temperature coefficient is 13460 cal. per g. molecule.

The temperature coefficient of the velocity of the reaction catalysed by the dehydrogenase has also been measured and $Q_{10°} = 1{\cdot}65$ at 30°. From this the calculated energy of activation of the citric dehydrogenase-citric acid compound is 8790 cal. per g. molecule.

These figures are contrasted with the corresponding ones for yeast saccharase and the significance of the contrast is discussed briefly.

My thanks are due to Professor T. Thunberg for advice concerning the use of the dehydrogenase, and to Professor J. B. S. Haldane for many valuable suggestions. I wish also to thank Sir F. G. Hopkins for much help and encouragement.

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XXV. THE AMINO-ACIDS OF GLUTENIN.

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The necessity for investigating new methods of protein analysis requires no emphasis. While the separation of the bases by the method of Kossel and Patten [1903], especially as recently modified by Vickery and Leavenworth [1928], and the precipitation of the dicarboxylic acids by calcium hydroxide and alcohol [Foreman, 1914] lead to nearly quantitative results, the isolation of the monoamino- and non-amino-monocarboyxlic acids by the usual ester method leaves much to be desired both from the point of view of purity of products and of yield. The different modifications of the ester method that have been devised have been successful in overcoming certain of its shortcomings. Preliminary extraction of the amino-acids as recommended by Dakin [1918] and the use of their lead salts for esterification adopted by Foreman [1919] lead to anhydrous preparations of the monoamino-acids which are therefore more readily esterified.

However in any separation of the amino-acids based on the fractionation of their esters certain serious drawbacks inherent in the method are bound to remain, *viz.* (i) loss of esters due to volatilisation and to decomposition during distillation, (ii) the difficulty of separating closely related substances which may appear in the same fraction of the distillate, and (iii) the presence of unmanageable syrups which hinder purification and crystallisation. In this connection Osborne's observations [1910] on the separation of a synthetic mixture of pure amino-acids—in which the recovery was only 72·3 %—are of significance. It is clear then that a successful method for the separation of amino-acids which does not involve the preparation and distillation of the esters would form a valuable contribution to the study of the amino-acid composition of the proteins.

Some of the results of a series of investigations carried out in this laboratory under the direction of the late Professor Schryver with the object of separating the amino-acids by taking advantage of the differences in the solubilities of their copper salts have already been published by Town [1928] and by Brazier [1930]. In the present paper the results of experiments on the separation of the copper salts of the amino-acids from wheat glutenin are described.

It has been possible to obtain higher yields of alanine, valine, proline and aspartic acid than those previously obtained by the ester method. Since the

completion of this work, the publication of which has for various reasons been considerably delayed, two of the increased values have been confirmed in other laboratories. Spörer and Kapfhammer [1930], using Reinecke salt for the precipitation of proline, have obtained a value (5·98 %) practically identical with that found in this investigation (6·15 %). Jones and Moeller [1928], who have used essentially the same procedure as that described in this paper for the isolation of the dicarboxylic acids, have found 2 % aspartic acid in close agreement with the value (1·85 %) now reported. Phenylalanine and leucine are found in the same fraction in both the copper salt and the ester method, and they have not been satisfactorily separated. The actual yields of these two substances are lower than those found by Osborne, but the use of an indirect method involving the oxidation of phenylalanine to benzoic acid has raised the value of phenylalanine to 2·75 % (ester method, 1·97 %).

Apart from the higher yields of certain individual amino-acids it has been possible to trace a large proportion of the nitrogen not accounted for in the products isolated. The nitrogen entirely unaccounted for amounts to only about 8 % of the total, a loss which can hardly be avoided considering the nature of the processes involved. By checking the amount of nitrogen lost by adsorption on various precipitates it has been found possible to gain some idea of the deficiencies that fall upon particular amino-acids. By this means also it has been possible to devise modifications of the method so as to obviate such losses in future hydrolyses.

Separation of the amino-acids is effected by taking advantage of the difference in solubility of their copper salts in water and in methyl alcohol. The extraction, under carefully regulated conditions, of the dry copper salts of the mixed amino-acids by these two solvents in succession gives three fractions.

(i) Copper salts soluble in methyl alcohol: *valine* and *proline*.

(ii) Copper salts insoluble in methyl alcohol but soluble in water under the conditions of the experiment: *glycine*, *alanine*, *aspartic acid*, *glutamic acid*, *arginine*, *histidine*, *lysine* and some *tyrosine*.

(iii) Copper salts insoluble in methyl alcohol and in water: *leucine*, *phenylalanine* and *aspartic acid*.

In each of the above fractions, especially (i) and (ii), a certain amount of unidentified matter was also present. The amino-acids in each fraction have been separated by taking advantage of the difference in solubility either of the acids themselves or of their derivatives in suitable solvents.

Experimental.

Preparation of the glutenin. Gluten, obtained by thorough washing of wheat flour, was repeatedly extracted with 73 % alcohol to remove the gliadin. The residue was dried at room temperature, ground to a fine powder and exhaustively extracted with alcohol. Excess of alcohol was removed at room

temperature with the help of a fan and the residue was dissolved in 0·2 % sodium hydroxide. The solution was filtered clear and precipitated by the addition of dilute acetic acid. The product was purified by reprecipitation from sodium hydroxide. It was then washed free from sodium acetate with dilute alcohol and finally dehydrated with acetone. The glutenin thus prepared contained 8·36 % water and 0·44 % ash, and, ash- and moisture-free, 17·02 % nitrogen.

Hydrolysis. 250 g. of glutenin equivalent to 228 g. of ash- and moisture-free protein and to 38·81 g. of nitrogen were hydrolysed by boiling with four times the weight of 25 % sulphuric acid under a reflux condenser for 24 hours. The cooled solution was diluted to 4 litres and the humin was filtered off, washed thoroughly with hot water and analysed for nitrogen. Ammonia was determined in a small aliquot by aeration. Most of the sulphuric acid was now precipitated by the addition of saturated baryta to the hydrolysate. The barium sulphate was repeatedly washed with hot water, then dried at 110° and washed again after powdering. A nitrogen determination on the barium sulphate showed that it retained 6·47 % of the total nitrogen of the protein. The filtrate and washings from the barium sulphate were concentrated to about 2 litres and the sulphuric acid was quantitatively removed. On further concentration to 750 cc. a precipitate of crude tyrosine separated which gave, after recrystallisation, 6·84 g. of the pure amino-acid.

Preparation of the dry copper salts. The filtrate from the tyrosine was heated on a water-bath in a large porcelain dish and treated with copper carbonate in small portions at a time. After the addition of a fairly large excess of copper carbonate the solution was evaporated to a thick syrup which was left on the water-bath for some hours. It was then taken up again in water and filtered from the excess of copper carbonate, and the latter was washed thoroughly with boiling water. The filtrate and washings were once more evaporated to a syrup with excess of copper carbonate to ensure complete conversion into the copper salts. After again taking up in water and filtering, the solution was evaporated to a thick syrup, allowed to cool and then stirred up with a liberal amount of acetone. After standing for a short time the acetone was poured off and replaced by a fresh quantity. On repeating this process three to four times a dark blue friable mass was obtained which was easily ground to a fine powder. This was filtered with suction and freed from acetone in a vacuum desiccator over sulphuric acid. It was then dried thoroughly at 110° for 24 hours. If the syrup is evaporated too far before granulation with acetone or if an appreciable amount of acetone is present in the mixture when placed in the drying oven hard cakes are obtained which are difficult to break up and impossible to dehydrate completely.

The thoroughly dried copper salts weighed 279 g. and contained 26·53 g. of nitrogen.

Fractionation of the copper salts.

(*a*) *Extraction with methyl alcohol.* The thoroughly dried and finely ground copper salts were shaken with three times their volume of dry methyl alcohol (freshly distilled from sodium) on a shaking machine. After about 2 hours the blue solution was filtered and fresh methyl alcohol was added to the residue. Seven extractions were usually sufficient to remove all soluble material. The combined extracts were filtered at the pump, evaporated to a syrup and the residue was granulated with acetone. After drying for one hour at 100° and taking up once more in methyl alcohol a small residue was usually obtained which was put back into the main fraction of copper salts. The solution when evaporated gave a residue completely soluble in methyl alcohol.

(*b*) *Extraction with water.* The residual copper salts were extracted four times successively with large volumes of water at 60°. The aqueous solutions were evaporated and the residue was dehydrated with acetone. The extraction was repeated on this residue, using very large volumes of warm water. Any insoluble residue was added to Fraction III consisting of the copper salts insoluble in both methyl alcohol and water. The three fractions were decomposed with hydrogen sulphide, I and II in aqueous solution, III in dilute sulphuric acid. The copper sulphide obtained in each case was very thoroughly washed and after drying was analysed for nitrogen. The filtrate and washings in each fraction were evaporated *in vacuo* to remove hydrogen sulphide and the nitrogen was determined in an aliquot in each case.

Fraction I. Amino-acids from copper salts soluble in methyl alcohol.

On evaporating the aqueous solution a pale brown sticky mass was obtained. This was thoroughly dehydrated by repeated addition of absolute alcohol and evaporation *in vacuo*. It was next exhaustively extracted with hot absolute alcohol. The insoluble residue was white and friable. The alcoholic extracts were evaporated and the residue was re-extracted with alcohol; a small amount of undissolved material was added to the main insoluble portion. This operation was repeated until the material obtained on evaporation was completely soluble in absolute alcohol.

Valine. The residues insoluble in ethyl alcohol were combined and reconverted into their copper salts. The material recovered by decomposition of the latter was entirely insoluble in alcohol. When recrystallised from water it had M.P. 313–315°, N 11·92 %, and was therefore valine. Yield 2·33 g. No alcohol-soluble zinc salt suggestive of hydroxyvaline [Schryver and Buston, 1926] could be obtained.

Proline. The material completely soluble in alcohol was recovered by evaporation and taken up in water. The solution was analysed for non-amino-nitrogen, diluted until the concentration of the latter was about 1 g. per 100 cc., brought to the boil and treated with a hot solution containing sufficient picric acid to combine with the non-amino-nitrogen present [Town,

1928]. After evaporating and cooling proline picrate crystallised out in the form of golden yellow needles. On further evaporation of the mother-liquor a second small crop was obtained. The proline picrate was washed at the pump with cold water and, after drying in the desiccator, was extracted with ether to remove excess of picric acid as well as another picrate found to be soluble in ether (see later).

The aqueous and ethereal filtrates from the proline picrate were freed from picric acid by means of sulphuric acid and ether. After the quantitative removal of sulphuric acid the solution was evaporated to a syrup, taken up in 95 % alcohol and treated with a saturated solution of cadmium chloride [Kapfhammer and Eck, 1927] which was cautiously added drop by drop until no further precipitation took place. The precipitate was filtered off and washed with cold 95 % alcohol until free from chloride, re-dissolved in water and freed from cadmium chloride (chloride precipitated by silver sulphate, and silver and cadmium by hydrogen sulphide). After the quantitative removal of sulphuric acid the solution was once more treated with picric acid. The combined proline picrate precipitates were recrystallised from water, when 42 g. of picrate melting at 148° were obtained corresponding to 14·02 g. of proline.

The mother-liquors from which proline had been recrystallised as the picrate and the residual proline had been precipitated as the cadmium chloride compound were freed quantitatively from all reagents and the aqueous solution was evaporated to a syrup. It contained 5·91 % of the total nitrogen. No crystallisation took place on long standing in the desiccator, and it was found impossible to obtain it in a dry state. No definite compound was isolated from this mixture, but it could be separated into two fractions, one forming a picrate soluble in ether, the other not giving a picrate.

Fraction II. Amino-acids from copper salts insoluble in methyl alcohol, but soluble in water.

Tyrosine. On evaporation of the amino-acid solution to about 600 cc. a small amount of tyrosine crystallised out. The weight of pure recrystallised material was 2·76 g., making with that previously isolated 9·6 g. of tyrosine in all.

Aspartic and glutamic acids. The solution was treated according to Kingston and Schryver's modification [1924] of Foreman's [1914] method for the isolation of the dicarboxylic acids. It was found that by adding baryta in a thin cream in fair excess and keeping the mixture vigorously stirred during the slow and gradual addition of alcohol the formation of a sticky precipitate could be avoided. The barium salts were re-dissolved in water and again precipitated with alcohol in the presence of excess of baryta. The filtrate and washings from this second precipitation were not added to the main filtrate but were examined separately. The precipitate was dissolved in water and the barium was removed quantitatively. The crystals obtained from this

solution on evaporation, though possessing the characteristic form of glutamic acid, were found to be impure and therefore they were fractionated by the procedure adoped by Dakin [1919]. Glutamic acid was separated in three successive crops as the hydrochloride, 72·65 g. of recrystallised hydrochloride, corresponding to 58·14 g. of glutamic acid, being obtained.

From the mother-liquors after removal of glutamic acid aspartic acid was separated as the lead salt [Dakin, 1919]. The yield of pure recrystallised aspartic acid was 0·52 g.

By treatment of the filtrate from the lead aspartate with sodium hydroxide and silver nitrate the silver salt of hydroxyglutamic acid was precipitated. It was not however found possible to obtain crystals of hydroxyglutamic acid even after long standing in the vacuum desiccator. The hard, dry mass thus obtained weighed 2·3 g. corresponding to 1 % of the weight of glutenin. It contained 0·54 % of ash. (N: found, 8·40 %; calc., 8·59 %.)

Glycine. The filtrate and washings obtained from the second precipitation of the barium dicarboxylates were freed from alcohol and barium. The solution gave a negative reaction with Millon's reagent showing the absence of tyrosine. A small precipitate was obtained with phosphotungstic acid. It was filtered off, decomposed and the solution so obtained was added to the main filtrate from the dicarboyxlic acid precipitation. The filtrate was freed from phosphotungstic acid and the solution was evaporated to dryness. The residue obtained consisted of glycine mixed with a certain amount of inorganic impurity. By recrystallisation 0·6 g. of pure glycine was obtained. (N: found, 18·57 %; calc., 18·67 %.) It was further identified by conversion into the picrate M.P. 189°.

Removal of the bases. In the main filtrate from the dicarboxylic acid precipitate arginine and histidine were precipitated as their silver salts by the method of Vickery and Leavenworth [1928]. The nitrogen in the solution obtained by decomposing the silver precipitate was 11·6 % of the total in good agreement with Osborne's [1910] value of 11·39 %. From the solution, after removal of arginine and histidine, lysine was separated in the usual way as picrate. After its removal the residual solution still contained 1 g. of nitrogen, which was not identified.

Alanine and glycine. The solution with the washings from the phosphotungstic acid precipitation above was freed from phosphotungstic and sulphuric acids and evaporated to 500 cc., and the amino-acids were converted into their barium carbamates by the method of Kingston and Schryver [1924]. The precipitate was dissolved in water at room temperature and freed from barium. On evaporation glycine (1·13 g.) crystallised out, making, with the amount already isolated, a total weight of 1·73 g.

The washings containing the soluble barium carbamate were boiled and the precipitated barium carbonate was filtered off. The filtrate, on evaporation, gave nearly pure alanine. The yield after recrystallisation from aqueous alcohol was 14·04 g. (N: found, 15·83 %; calc., 15·73 %.) In view of the difficulty of effecting a complete separation of alanine and glycine, either by

the ester method or by the formation of their calcium chloride compounds [Pfeiffer and Wittka, 1915] it may be pointed out that the procedure described above gives a very clean and satisfactory separation of these two substances.

Fraction III. Amino-acids from copper salts insoluble in methyl alcohol and water.

The copper salts in this fraction were brought into solution by suspending them in water and adding small quantities of sulphuric acid from time to time as necessary, and were decomposed as before.

The alcohol-insoluble barium salts from this fraction yielded, when decomposed, *aspartic acid.* (Yield after recrystallisation from water 3·7 g., making, in all, 4·22 g. or 1·85 % of the weight of glutenin.)

Leucine and phenylalanine. The main filtrate from the barium aspartate was freed from alcohol and barium and evaporated to a small volume on the water-bath. Most of the material crystallised out in flakes which were filtered off and washed with cold water. On evaporating the mother-liquor and washings a small second crop of crystals was obtained. The dry crystals contained 10·03 % N. The mother-liquor, on evaporation to dryness, gave a crystalline powder, the nitrogen content of which (10·01 %) showed it to be of similar composition to the earlier crops of crystals. The method of fractionation employed suggested that this material was a mixture of leucine and phenylalanine. Separation of the two amino-acids however was only partially successful. By repeated fractional crystallisation 8·5 g. of nearly pure leucine was obtained. (M.P. 280–285°. N: found, 10·4 %; calc., 10·69 %.) The residual mixture (12 g. containing 9·76 % nitrogen) was converted into the zinc salt [Brazier, 1930]. From the water-soluble portion of the dried salts, after removal of zinc, 3·45 g. of crude phenylalanine was obtained. (N: found, 8·86 %; calc., 8·49 %.)

The residue insoluble in cold water was made alkaline with a little barium hydroxide and decomposed with hydrogen sulphide. On evaporating the solution 8·5 g. of material having 10·13 % N crystallised out. As it was found impossible to effect any further separation either by crystallisation or by the use of the zinc salts the phenylalanine content was determined indirectly by oxidation to benzoic acid [Kollmann, 1928]. The mixture was oxidised by boiling for 6 hours with Beckmann's solution; the benzoic acid, together with the fatty acids formed, were extracted in a continuous extractor with ether, and after evaporation of the ether the benzoic acid was separated by washing the residue with water previously saturated with benzoic acid. The benzoic acid was dried to constant weight at 60°. Duplicate experiments gave the following results: 1·505 g. of the mixture yielded 0·3476 g. of benzoic acid, equivalent to 23·33 % of phenylalanine; 1 g. gave 0·1645 g. of benzoic acid equivalent to 23·66 % of phenylalanine. 0·5 g. of pure phenylalanine gave 0·3476 g. of benzoic acid corresponding to a yield of 94 %. The mixture thus contained 23·5 % phenylalanine and 76·5 % leucine. Calculated from the nitrogen

content the figures are 25·5 % and 74·5 % respectively. As the discrepancies are within the limits of error of the oxidation method it seems legitimate to assume that the mixture consisted only of leucine and phenylalanine and to adopt the latter of the above sets of figures as representing its composition. On this assumption the gross value for phenylalanine is 2·75 % and for leucine 6·3 % of the glutenin.

The following tables summarise the results obtained. Table I gives the distribution of nitrogen in the main fractions at the stage prior to the liberation of the amino-acids from their copper salts. Table II gives the percentages of the different products isolated, both in terms of total nitrogen and of total weight of protein taken, together with the values obtained by previous workers for comparison. Column 2 of Table II gives an indication of the losses encountered; those in precipitates are in some cases ascribed to particular amino-acids. It is seen, for example, that the value for glycine is at least 20 %, for alanine 5 % and for aspartic acid 10 % too low. By combining the highest values from the present analysis with those obtained by previous workers 70·98 % of the protein is now accounted for in the products of hydrolysis. In the case of the monoamino-acids, with which this method is chiefly concerned, the value 22·14 % given by the ester method has been raised to 27·34 %.

Table I. *Distribution of nitrogen in different fractions.*

	Nitrogen in g.	Nitrogen %
Humin	0·45	1·17
In main barium sulphate precipitate	2·51	6·46
In solution	35·06	90·36
Loss	0·79	2·01
Total	38·81	100·0
In tyrosine isolated before forming copper salts ...	0·53	1·36
In ammonia	7·41	19·10
In excess copper carbonate	0·26	0·67
In copper salts	26·53	68·38
Loss	0·33	0·85
Total	35·06	90·36
In Fraction I of the copper salts	4·64	11·96
In Fraction II	17·75	45·74
In Fraction III	3·17	8·17
Loss	0·97	2·51
Total	26·53	68·38

Discussion.

The following advantages can be claimed for the method of separation described.

1. By avoiding the losses inherent in the ester method higher yields of most of the monoamino-acids have been obtained, notably in the case of proline, valine, alanine and phenylalanine.

Table II.

	% of total nitrogen. Copper salt method		% of weight of protein	
	In products isolated	In precipitates and unidentified syrups	Copper salt method	Other methods
Humin	—	1·17	—	—
Main barium sulphate precipitate ...	—	6·46	—	—
Ammonia	19·1	—	3·97	4·01[1]
Excess copper carbonate...	—	0·67	—	—
Tyrosine (both fractions)...	1·91	—	4·20	4·25[1]
Fraction I:				
Copper sulphide	—	0·53	—	—
Valine	0·72	—	1·02	0·2[1]
Proline	4·39	—	6·15	5·98[2]
Unidentified syrup	—	5·91	—	—
Fraction II:				
Copper sulphide	—	1·60	—	—
Barium sulphate from first precipitation	—	0·22	—	—
Glutamic acid	14·78	—	26·49	25·7[3]
Hydroxyglutamic acid	0·5	—	1·00	1·8[4]
Lead hydroxide and sulphate	—	0·07	—	—
Arginine and histidine	11·60	—	—	—
Silver and barium precipitates	—	0·25	—	—
Lysine and unidentified matter	—	6·42	—	—
Barium phosphotungstate	—	0·20	—	—
Glycine (from Fractions I and II) ...	0·83	—	0·76	0·89[1]
Barium sulphate from carbamate ...	—	0·19	—	—
Alanine	5·68	—	6·16	4·65[1]
Barium sulphate	—	0·29	—	—
Fraction III:				
Copper sulphide	—	1·05	—	—
Barium sulphate	—	0·09	—	—
Aspartic acid	1·15	—	1·85	2·0[3]
Leucine	3·96	—	6·3	5·95[1]
Phenylalanine	1·37	—	2·75	1·97[1]

[1] Osborne and Clapp [1906].
[2] Spörer and Kapfhammer [1930].
[3] Jones and Moeller [1928].
[4] Dakin [1919].

2. The indeterminate losses of the ester method are replaced by definite losses in precipitates which can be readily determined. Thus some idea is gained of the deficiencies falling on individual amino-acids.

3. The fractionation of the copper salts gives rise to groups of amino-acids which are more amenable to separation than are the fractions obtained by conversion into the esters. Thus alanine, valine and the leucines, which in the ester method form one group extremely difficult to separate, are here obtained in three fractions.

4. The amino-acids set free from the copper salts are, as a rule, free from contaminating syrupy material and therefore readily crystallisable.

On the other hand, the procedure described here, though adopted after

many preliminary separations on rather different lines, still suffers from the following drawbacks.

(*a*) The considerable loss of the hydrolysis products in the first barium sulphate precipitate.

(*b*) The difficulty of separating the copper salts of aspartic and glutamic acids in the presence of large amounts of the latter.

(*c*) The inability to isolate serine and cystine. In one preliminary experiment the dicarboxylic acids and the diamino-acids were first removed, the former as the barium salts, the latter by means of phosphotungstic acid, prior to conversion into copper salts. This procedure was abandoned on the ground that the barium dicarboxylates carried traces of other amino-acids with them and the basic phosphotungstates were not completely insoluble. Later experience has however shown that the preliminary removal of these two groups of amino-acids would considerably simplify the fractionation of the copper salts of the monoamino-acids and in future work this modification is recommended.

Summary.

1. The hydrolysis products of glutenin have been fractionated by means of their copper salts.

2. Higher yields of many of the monoamino-acids have been obtained.

3. Possible improvements in the method are indicated.

Grateful acknowledgment is made to the late Professor S. B. Schryver, at whose suggestion this work was undertaken.

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XXVI. STUDIES ON THE GUMS.
II. TRAGACANTHIN—THE SOLUBLE CONSTITUENT OF GUM TRAGACANTH.

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The literature on the subject of gum tragacanth, like that of the gums in general, is fragmentary and confused. The first work of value is that of Ogle [1900] who stated that this gum is not a simple substance, but contains 36 % of a water-soluble carbohydrate constituent and 42 % of an insoluble one, which swells up in water. The soluble constituent was stated to give a product on precipitation with alcohol, different from arabin (acid gum arabic). Widstoe and Tollens [1900] treated gum tragacanth as a simple substance, from which they obtained on hydrolysis the sugars, fucose and arabinose, and from another sample, xylose and arabinose. In the same year Hilger and Dreyfus [1900] stated that this gum contained 15–22 % galactose, estimated as mucic acid, and 30–42 % arabinose. Tollens [1901], as a result of ultimate analyses, formed the opinion that this gum contained carboxyl groups, a view contrary to that held by Hilger and Dreyfus. O'Sullivan [1901] gave an account of some very detailed work on the composition of this gum. He opens with the statement that gum tragacanth had been held to be a mixture of "cellulose, starch, bassorin, a gum-like arabin, a little nitrogenous matter, sugar, and ash." The soluble constituent "gum-like arabin" he concludes to be a mixture of poly-arabinon-trigalactan-geddic acids and gives for it the formula

$$x\,[C_{10}H_{16}O_8 . 3C_{12}H_{20}O_{10} . C_{23}H_{38}O_{22}].$$

More recently von Fellenberg [1914] states that whereas the bassorin or insoluble portion is methoxylated to the extent of 5·38 %, the water-soluble constituent has no ester group of this type.

It seems, strangely enough, that a name has never been given to this constituent of gum tragacanth soluble in water, and it is proposed to term it tragacanthin.

Experimental.

Gum tragacanth on addition of water swells enormously, since the water-insoluble form, bassorin, constituting some 60–70 % of the gum, gives a very bulky jelly. O'Sullivan [1901] stated that he effected a separation of the soluble gum from the bassorin by means of fractional precipitation with alcohol. A solution of gum tragacanth in water was concentrated until a gelatinous scum began to form on the surface of the liquid. Dilute alcohol was added

gradually until a bulky and curdy precipitate came down, which was stated to be bassorin. From the alcoholic filtrate, on evaporation and addition of strong alcohol, a further precipitate was obtained, claimed to be the tragacanthin. This he fractionated several times; but nevertheless it cannot be held to be an ideal method of separation. Some more reliable method was sought for in this work, and many methods both physical and chemical were tried. For a time a stream-line filter works admirably with a suitable grade of paper, but unless it is very large it chokes before any considerable quantity has filtered. Finally, filtration on very large fluted filter-papers was adopted. This was an extremely laborious method since filtration was slow, and since a concentration of the gum higher than about 0·1 % could not be employed. The filter-papers have frequently to be changed; but nevertheless such a method makes it absolutely certain that the product obtained is tragacanthin only. The very dilute filtrate was concentrated under reduced pressure to a small bulk, and several volumes of 95 % alcohol were added, together with a small quantity of hydrochloric acid. A stringy precipitate formed, not unlike many pectin preparations in appearance. This was filtered off, dissolved in water, and again precipitated with acid alcohol. After several such reprecipitations the product was dried in absolute alcohol and finally in a desiccator over phosphorus pentoxide.

A satisfactory separation was also effected in the following manner. The solution of the gum was first made alkaline and then just slightly acid by the addition of acetic acid, whereupon the physical condition of the bassorin is somewhat altered, and it appears as large gelatinous flocks. These were removed by running the liquid through a super-centrifuge. The effluent from the centrifuge was allowed to stand and the clear supernatant liquid finally filtered through filter-paper as before. The final product in each case was a fine white powder very readily soluble in cold water.

A portion was hydrolysed by boiling for one hour with sulphuric acid, and the solution examined for sugars after the removal of the acid by baryta. Three volumes of hot alcohol were added to the hydrolysate to remove incompletely hydrolysed gum and the filtrate concentrated under reduced pressure to a thin syrup which was then utilised for the tests. Contrary to the observation of O'Sullivan [1901] galactose was not found, for only a trace of mucic acid remained on oxidation with nitric acid. The only sugar that could be detected was arabinose, which was present in some quantity. This was identified by means of its diphenylhydrazone.

The yield of carbon dioxide as a measure of uronic acid content was determined in the usual way.

Tragacanthin. Sample I	Ash	0·87 %
Yield of CO_2 on ash-free basis	(i)	12·65 %
	(ii)	12·75 %

Mean value 12·70 % corresponding to 50·8 % uronic acid anhydride.

It is not possible to be definite as to the nature of the uronic acid, though there is some evidence for supposing that it is galacturonic.

The yield of furfuraldehyde was next determined so that a figure could be obtained by calculation for the pentose present in the molecule.

Yield of furfuraldehyde on ash-free basis... ...	(i)	31·26 %
	(ii)	31·42 %
Mean value		31·34 %

Since uronic acids themselves yield 16·66 % of furfuraldehyde, the 50·8 % uronic acid anhydride present in the molecule is responsible for the yield of 8·48 % furfuraldehyde, leaving the balance 22·88 % as due to pentose. This, calculated as anhydroarabinose, is 43·12 %.

Since no sugar other than arabinose could be detected in the hydrolysis liquid, it seems likely that tragacanthin consists solely of uronic acid and arabinose, though it is only possible to account for 94 % of the molecule in this way. There is a great divergence between this conclusion and that of O'Sullivan [1901], who considered tragacanthin to be a complex poly-arabinon-trigalactan-geddic acid, yielding on hydrolysis, arabinose, galactose, and geddic acid, an isomer of arabic acid also obtained by him from certain constituents of gedda gum. It seems likely that the preparation obtained by O'Sullivan contained some bassorin, which would account for the presence of galactan units.

To throw further light on the constitution of this gum several hydrolyses were carried out, and an examination of the hydrolysis products was undertaken. A portion of tragacanthin was dissolved in 3 % sulphuric acid and boiled under reflux for the period stated. It was then nearly neutralised with barium carbonate while still hot, filtered and poured into hot alcohol. A precipitate rapidly settled out and the supernatant liquid was poured off before it cooled. The precipitate was redissolved, filtered and reprecipitated with hot alcohol as before. This process was repeated several times in each case to ensure the removal of all free sugar. Finally it was dried with absolute alcohol and over phosphorus pentoxide.

The following analytical figures were obtained for the various preparations:

Table I. *Hydrolysis of tragacanthin with 3 % sulphuric acid.*

Preparation	Tragacanthin sample I %	30 minutes' hydrolysis %	60 minutes' hydrolysis %	90 minutes' hydrolysis %
Ash	0·87	1·04	2·87	3·70
CO_2 yield	12·70	18·83	19·27	20·28
Furfuraldehyde yield	31·34	23·76	23·18	22·20
Uronic acid anhydride	50·80	75·32	77·08	81·12
Furfuraldehyde from uronic acid	8·46	12·55	12·84	13·50
Furfuraldehyde from pentose	22·88	11·21	10·34	8·70
Anhydroarabinose	43·10	20·89	19·27	16·25

The composition of the hydrolysis products is illustrated in Fig. 1. It will be noted that during the initial period of hydrolysis the arabinose content

diminishes rapidly, and the uronic acid increases proportionately. After 30 minutes, however, very little more arabinose is removed during a further period of 30 minutes, and even up to 90 minutes the product remains of much the same order. After this period hydrolysis soon becomes complete. These

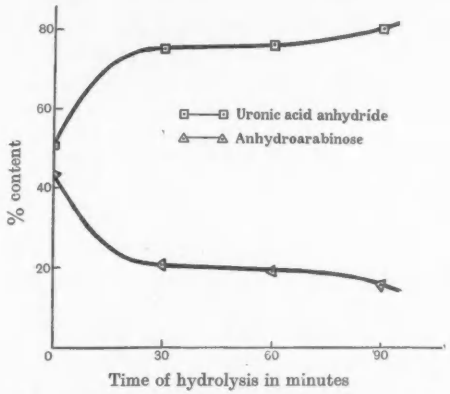
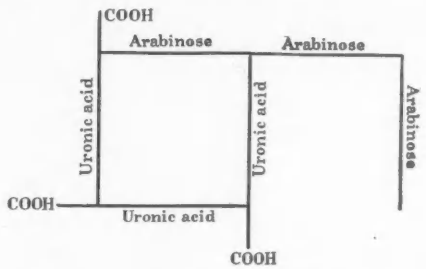


Fig. 1. Hydrolysis of tragacanthin with 3 % H_2SO_4.

results throw a considerable amount of light on the constitution of the tragacanthin molecule. It is clear that the uronic acid and a portion of the arabinose form a more resistant nucleus acid, probably linked in the form of a ring. Attached to this by a glucosidic linkage must be the remaining arabinose, which is thus easily removed on hydrolysis. A simple arrangement agreeing fairly closely with the figures experimentally obtained is a nucleus acid consisting of three molecules of uronic acid linked with one of arabinose to form a four-membered ring, attached to which is a side chain of two molecules of arabinose, as illustrated diagrammatically below.



Such a nucleus would have a composition of approximately 20·0 % anhydroarabinose and 80·0 % uronic acid anhydride, which is not dissimilar from that corresponding with the figures obtained after hydrolysis for a period of 1 hour. It is, of course, not claimed that the constitution of this gum is

represented above, but the evidence obtained from a study of the course of hydrolysis strongly suggests this general type of arrangement. A study of gum arabic [Norman, 1929] on similar lines led to the conclusion that it also contains a nucleus acid, resistant to mild hydrolysis, to which are attached arabinose units by a type of linkage easily broken. A similar type of arrangement has just been described for mesquite gum by Anderson and Otis [1930]. In this substance they find a resistant nucleus containing three units of galactose and one of methoxyglycuronic acid, to which are attached through a dicarbonyl linkage, four units of arabinose. It is probable therefore that this general arrangement may be found to hold for other members of this group of substances. Bassorin, which is very much more complex in structure than tragacanthin seems to be similarly constituted.

Summary.

1. Tragacanthin, the soluble constituent in gum tragacanth, may be separated by ordinary filtration in extreme dilution.

2. Uronic acid units are found to be present and to constitute about one-half of the molecule. Arabinose was the only sugar found; no galactose could be detected.

3. Hydrolysis products were prepared, the analytical figures for which give rise to the suggestion that a portion of the arabinose is united to the uronic acid to form a resistant nucleus, and the residue attached by glucosidic linkage, and therefore easily removable.

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XXVII. HYDROGENASE: A BACTERIAL ENZYME ACTIVATING MOLECULAR HYDROGEN.

I. THE PROPERTIES OF THE ENZYME.

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Biochemists are now accustomed to regard the transfer of hydrogen as an essential step in biological oxidations; such a view involves the conception of some enzymic mechanism for rendering active or unstable the molecule from which the hydrogen is transferred. No such enzyme acting on molecular hydrogen has so far been described, though several inorganic catalysts are known which function in this way. It is nevertheless almost certain that such an enzyme exists, as organisms producing molecular hydrogen presumably have such a catalyst; moreover bacteria have long been known which are capable of oxidising hydrogen gas by molecular oxygen and living autotrophically on the energy thus liberated. The earliest example of such an organism was *B. pantotrophus* (*Hydrogenomonas pantotropha*) isolated by Kaserer [1906]. Later other workers isolated a variety of species having the same characteristic [Niklewski, 1914; Grohmann, 1924]. It was subsequently discovered that in certain of these species nitrate can replace oxygen, so that an anaerobic autotrophic development occurs [Niklewski, 1914]. In all these cases the organisms are facultatively autotrophic, the oxidation of hydrogen playing an essential part in their metabolism.

A suggestion that these early observations come into line with recent work on the dehydrogenases of bacteria is contained in the recent work of Tausz and Donath [1930], who showed that a culture of *Bact. aliphaticum liquefaciens* grown autotrophically on hydrogen, oxygen and carbon dioxide is capable of reducing methylene blue when a stream of hydrogen is led through the apparatus, but not when nitrogen replaces the hydrogen.

Experimental.

During the study of anaerobic fermentations of fatty acids to methane by mixed cultures from river mud, a culture was obtained which (*a*) reduced sulphate to sulphide, and (*b*) decomposed formate quantitatively to methane, carbon dioxide and water. The same culture was also found to synthesise

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methane from a mixture of carbon dioxide and hydrogen and simultaneously to reduce sulphate to sulphide at the expense of hydrogen [*cf.* Söhngen, 1910]. This led to the conception that carbon dioxide and sulphate were acting as hydrogen acceptors in a system where molecular hydrogen was the hydrogen donator, and it seemed likely that bacteria were present in the mixed culture capable of activating molecular hydrogen. We therefore centrifuged a mixed culture which had been grown for some 20 subcultures on formate as sole source of carbon, and tested the washed suspension in Thunberg tubes with methylene blue and gaseous hydrogen. For this purpose hydrogen from a Kipp's apparatus was passed through three wash-bottles containing silver nitrate (for the removal of traces of volatile arsenic compounds), pyrogallol and water; the last bottle was connected with a 3-way tap (oblique bore) so that the Thunberg tube could be alternately evacuated and filled with hydrogen. The tube was evacuated thoroughly to remove dissolved oxygen, then filled with hydrogen and again evacuated and filled; finally the tap was closed and the tube was placed in a bath at 45° and shaken for 1 minute to ensure saturation of the liquid with the gas; the reduction time was measured.

Result:

	Reduction time (mins.)
1 cc. 1/25,000 methylene blue, 1 cc. phosphate buffer p_H 8·0, 1 cc. bacterial suspension, in hydrogen	15
1 cc. 1/25,000 methylene blue, 1 cc. phosphate buffer p_H 8·0, 1 cc. bacterial suspension, *in vacuo*	> 70
Control with boiled suspension in hydrogen	> 70

Having shown that our mixed culture contained organisms capable of reducing by means of molecular hydrogen, we diluted and plated on ordinary broth agar and incubated the plates anaerobically. We picked off a number of colonies, grew the corresponding cultures on broth, centrifuged and washed the organisms, and tested the suspensions as above with hydrogen and methylene blue. In these and all subsequent experiments 1 cc. of 1/5000 methylene blue in a total volume of 4 cc. was used, and the time was taken when reduction had proceeded to 75 %, because the rate of reduction appears to fall off after that point.

Result:

	Reduction time (mins.)	
Culture No.	With hydrogen	Without hydrogen
70	5½	> 120
74	> 120	> 120
79	> 120	> 120
80	5½	> 120

This shows that there is a clearly marked difference between organisms having the property of reducing by means of molecular hydrogen and those lacking the power. Culture 70 was re-plated and gave the strain known as 111.

Characteristics of organism 111. This organism is a short, motile, non-sporing, Gram-negative bacillus, growing both aerobically and anaerobically.

Sown into peptone medium with 1 % of various sugars, according to the usual bacteriological practice, the following results were obtained:

Sugar	Acid	Gas
Glucose	+	+
Fructose	+	+
Mannitol	−	+
Sucrose	−	−
Maltose	−	+
Lactose	+	+
Salicin	−	−

Other characteristics are (1) acid clot in litmus milk, (2) no liquefaction of gelatin, (3) no production of indole, (4) negative Voges and Proskauer test. We are therefore assigning this organism to the *coli-typhosus* group; its properties as far as they have been investigated agree with those of *B. formicus* (*Escherichia formica*), but its identity cannot be considered proved.

Hydrogenase. It is clear from the experiments cited already that we are dealing with a bacterial enzyme comparable with the dehydrogenases, the substrate in this case being molecular hydrogen. We must assume that the hydrogen is in some way activated, and this activation can be conveniently expressed $H_2 \rightleftharpoons 2H$ without implying anything about the nature of the reaction. In order to conform with the accepted terminology we suggest that this enzyme be called "hydrogenase."

Distribution of hydrogenase. The presence of this enzyme in what appears to be a common bacterial species made us suspect that it might be found in other well-known organisms. Various strains were grown on broth medium, centrifuged and washed, and tested in vacuum tubes in the usual way, and the following results were obtained. (To get a rough comparison between the activities of the different species, the suspensions were diluted to the same opacity.)

Strain	Reduction time (mins.) With H_2	Reduction time (mins.) Without H_2	Hydrogenase
Bact. coli (Escherich)	8½	> 120	+
Bact. coli (Houston 1)	11¼	75	+
Bact. coli (Houston 2)	8	> 120	+
Bact. coli (Houston 3)	8½	> 120	+
Bact. acidi lactici	9¼	> 150	+
Pseudomon. pyocyanea	65	63	−
Chromobact. prodigiosum	> 60	> 60	−
Bac. megatherium	> 120	> 120	−
Bact. lactis aerogenes	9½	15½	?
Clostr. sporogenes	> 150	> 150	−
Bac. subtilis	> 120	> 120	−
Bact. alcaligenes	27½	28	−

The relation of these results to other known facts will be discussed later.

The unexpected discovery of hydrogenase in a number of widely differing bacterial species led us to look for it elsewhere; baker's yeast and sheep's heart muscle, both normally active with regard to other dehydrogenases, were completely lacking in hydrogenase.

Properties of hydrogenase.

(*a*) *Effect of pressure of hydrogen.* From electrode potential measurements it has been shown that methylene blue 50 % reduced would be in equilibrium with 10^{-15} atmospheres of hydrogen at p_H 7·0 and 30°; so that at pressures far too small for measurement we may expect methylene blue to be completely reduced provided the molecular hydrogen is activated, as it is by platinum black or by hydrogenase. This complete reduction at very low pressures naturally cannot be verified experimentally. Apart from the effect of pressure in deciding whether the methylene blue will be reduced or not, we should expect variation of pressure to affect the velocity of reduction, the upper limit of pressure at which this effect becomes noticeable being decided by the affinity of hydrogenase for hydrogen. Actually we have been unable to measure this affinity owing to other complications. In the first place, even at pressures still capable of manometric measurement, the amount of hydrogen in solution in the liquid is insufficient stoichiometrically to reduce the methylene blue present in the tube, so that the rate of diffusion of hydrogen into the liquid becomes the limiting factor; in the second place, at still lower pressures, the total amount of hydrogen in the vacuum tube is insufficient to effect the complete reduction of the methylene blue. By lowering the methylene blue concentration these difficulties could be to some extent removed, but a limit is imposed by the experimental difficulty of measuring the reduction time of very small quantities of methylene blue.

The rates of reduction of the dye were measured at different pressures of hydrogen, using the same apparatus as that described previously, except that a manometer was inserted between the 3-way tap and the pump, and a tap between the manometer and the pump. But this means the vacuum tubes could be filled with hydrogen at any desired pressure.

Two curves were obtained, one using a concentration of 1/20,000 methylene

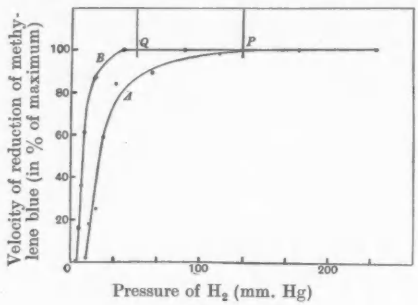

Fig. 1.

blue (Fig. 1, curve *A*), the other using 1/50,000 (curve *B*). From the solubility of hydrogen at 45° and the molecular weight of methylene blue it is calculated

that at a pressure of 130 mm. (*P*) just sufficient hydrogen is dissolved to reduce the higher concentration of methylene blue, and that at 50 mm. (*Q*) there is enough dissolved for the reduction of the lower concentration (*cf.* curves *A* and *B*). Hence the pressure-velocity curves give no measure of the affinity of the enzyme for its substrate, but serve to show that by working at a pressure of one atmosphere we are certain that the enzyme is saturated.

(*b*) *Effect of hydrogen ion concentration.* The relation between hydrogen ion concentration and velocity of reduction of methylene blue has been determined in the case of two organisms, viz. *Bact. coli* (Escherich) and strain 111. The results are given in Figs. 2 and 3, each compiled from the results of two separate experiments.

Alcock and Cook [personal communication] have determined the p_H curves of several bacterial dehydrogenases, and they find that after the characteristic increase of velocity up to a maximum at p_H 6·0 the velocity remains constant up to p_H 9·0, *i.e.* until irreversible destruction of the enzyme takes place in

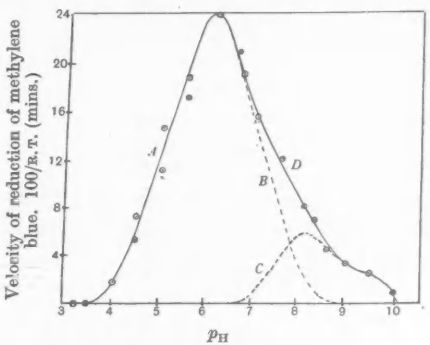

Fig. 2. *Bact. coli* (Escherich).

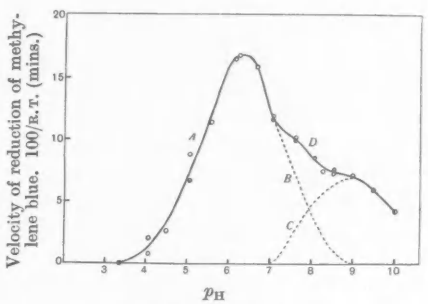

Fig. 3. Strain 111.

the alkaline region. In this respect hydrogenase differs very greatly from these dehydrogenases. Bernheim however [1928] described an aldehyde oxidase

from potato whose p_H-activity curve with respect to methylene blue showed a very clear peak with its optimum at p_H 6·8.

The shoulder on the descending part of the curve is difficult to explain; it is possible to analyse it into two dissociation residue curves, as shown by the dotted lines in the diagram. The ascending part of the curve *A* is identical in the case of the two organisms; the descending line *B* is obtained by drawing a curve symmetrical with *A*; the points on the line *C* are obtained by subtracting the values on the hypothetical curve *B* from the corresponding points on the experimental curve *D*. Whether this may be regarded as evidence for the existence of two hydrogenases having different p_H optima is a matter we are not prepared to discuss; it is noteworthy, however, that the optimum on the first peak is at about p_H 6·3 and that on the second at about p_H 8·5 in both organisms.

(*c*) *Effect of cyanide.* There is obviously an analogy between the activation of hydrogen by hydrogenase and that of the hydrogen atoms of carbon compounds by dehydrogenases. This similarity is borne out by the action of potassium cyanide, as the following experiment shows.

In order to show the effect of cyanide on the rate of reduction of methylene blue in a vacuum tube it is necessary to guard against the loss of hydrogen cyanide during the evacuation. For this purpose vacuum tubes with hollow stoppers were employed, the potassium cyanide solution being placed in the stopper at p_H 12; the contents of the tubes themselves were strongly buffered at p_H 6·0, and it was found by trial that when the contents of the stopper were mixed with those of the tube the p_H of the resulting mixture was not above 6·5, *i.e.* was still within the optimum range.

Results:

Concentration of KCN	Reduction time (mins.)
0	7
M/800	5
M/240	4¼
M/ 80	4
M/ 24	8

(*d*) *Effect of narcotics.* Keilin [1929] has shown that whereas the inhibition of respiration produced by cyanide is due to inhibition of the indophenol oxidase, that produced by urethane is to be attributed to inhibition of the reducing systems of the cell, *viz.* the dehydrogenases. The following figures show that hydrogenase is also inhibited by urethane:

Conc. of urethane (%)	Reduction time (mins.)	% inhibition
0	4¼	0
0·025	7¼	41
0·25	8¾	51·5
2·5	11¾	64

In its reactions to cyanide and urethane hydrogenase behaves as a typical dehydrogenase.

Reduction of other hydrogen acceptors.

One would expect that any substance that can be reduced by a given organism with the aid of reducing substances will also be reduced by it with molecular hydrogen provided that the organism possesses hydrogenase. This has been tested by means of the Barcroft differential manometer in the cases of *Bact. coli* and strain 111.

Reduction of molecular oxygen. For this purpose the right-hand cup of the apparatus was separately evacuated and filled with a gas mixture containing 20 % oxygen and 80 % hydrogen. The left-hand cup remained filled with air. Each cup contained 3 cc. of bacterial suspension in phosphate buffer. A typical result is shown in Fig. 4, curve 1. It is seen that in this case the activity of the enzyme falls off rapidly; we first suspected the formation of hydrogen peroxide but failed to demonstrate it by the peroxidase and guaiacum test. It seems however that mere shaking with air inactivates the enzyme; this we demonstrated by removing the contents of the left-hand Barcroft cup and placing it in a vacuum tube with methylene blue and adding hydrogen in the usual way. The reduction time was > 1 hour, while that of the untreated control was 7 mins.

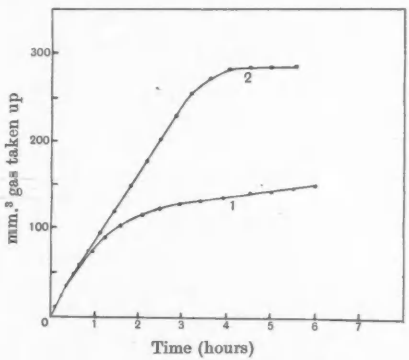

Fig. 4. 1. H_2+O_2. 2. Nitrate in H_2.

Reduction of nitrate. For these experiments both cups of the Barcroft apparatus were completely evacuated and filled with hydrogen. Potassium nitrate (M/1000–M/300) was present only in cup 2. The uptake of hydrogen proceeded linearly, but never attained completion; Fig. 4, curve 2, gives a typical result, the hydrogen here taken up corresponding to 53 % reduction of the nitrate to nitrite. Estimation of the nitrite at the end of the experiment, however, showed that the nitrate was completely reduced. A further addition of nitrate (in a companion experiment) showed that reduction again proceeded to the same extent, though slightly more slowly, and the same was true of a third and fourth addition (Fig. 5). The explanation of this phenomenon was obtained by carrying out parallel experiments in vacuum tubes where no

hydrogen was present. Here it was found that the nitrate was also reduced by the donators of the cell alone; hence in the Barcroft cup we have the result of a competition for the nitrate between the donators of the cell and the hydrogen.

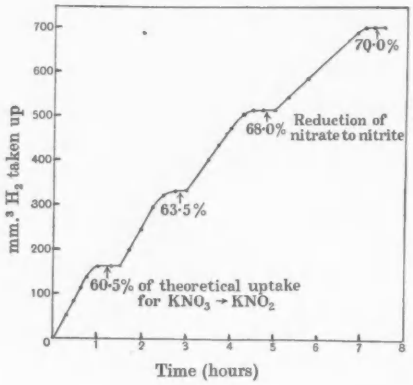

Fig. 5.

Exp.		Nitrate used (*M*)	Nitrite found (*M*)	H_2 uptake (% of theory for $NO_3' \rightarrow NO_2'$)
1	In hydrogen	0·0040	0·0044	41·0
2	,,	,,	0·0043	33·0
3	,,	0·0080	0·0070	39·5
4	,,	,,	0·0072	42·5
5	*In vacuo*	0·0040	0·0042	—
6	,,	,,	0·0037	—
7	,,	,,	0·0040	—

We attempted to obtain a reduction of nitrate by hydrogen and hydrogenase dissociated from reductions by cell substances; for this purpose we incubated the cell suspension with a 0·3 % solution of potassium nitrate for varying periods; the resulting nitrite was then removed by centrifuging and the suspension was tested as before with fresh nitrate in the Barcroft apparatus. This was however unsuccessful as the preliminary treatment so damaged the hydrogenase that the rate was greatly reduced and the hydrogen uptake was even less complete than when the untreated suspension was used.

Reduction of fumarate. The reduction of fumarate by hydrogen was shown in the same way as that of nitrate, *M*/30 to *M*/120 fumarate being used in place of nitrate. Hydrogen was taken up linearly but the uptake ceased when only about 30 to 60 % of that required for the complete reduction to succinate had disappeared. This incomplete uptake is probably due to the same cause as in the case of nitrate, *i.e.* to a part of it being reduced by the hydrogen donators of the cell; possibly also the anaerobic decomposition of fumarate occurring alongside the reduction may be a contributory cause.

The reduction of bacterial substance. It is well known that bacteria, in common with other living tissues, effect two kinds of oxidation, an endogenous

oxygen uptake in which the cell substance alone is concerned [Callow, 1924] and a much more rapid exogenous uptake when an oxidisable substance is added [Cook and Stephenson, 1928]. We therefore sought to discover whether, in addition to the exogenous reductions already demonstrated as the result of the action of hydrogenase, an endogenous reduction of cell material could also occur. For this purpose 3 cc. of a suspension of *Bact. coli* in buffer at p_{H_2} 6·5 was placed in the right-hand Barcroft cup and 3 cc. of buffer alone in the left-hand cup; both cups were evacuated and filled with hydrogen; no hydrogen uptake was observed. We therefore conclude that the cell has no store of hydrogen acceptors, and therefore no endogenous reduction occurs comparable with cell respiration.

The relation of hydrogenase to dehydrogenases and its function in cell metabolism.

From its general character it seems justifiable to regard hydrogenase as an enzyme closely related to the dehydrogenase class. It also seems likely that the enzyme which catalyses the change of molecular to activated hydrogen catalyses also the reverse reaction, *viz.* the production of molecular hydrogen from activated hydrogen atoms, that is, the reversible reaction, $H_2 \rightleftharpoons 2H$, should be catalysed in both directions. Previous studies by one of us [Stickland, 1929] have made it clear that the production of molecular hydrogen from formate is the work of two enzymes; it was there shown (1) that non-gas-producing organisms (*e.g. Bact. typhosum*) may possess an active formic dehydrogenase, (2) that in the case of *Bact. coli*, which possesses the double mechanism, treatment with trypsin augments the dehydrogenase and destroys the mechanism producing gaseous hydrogen. We therefore tentatively suggest that the production of molecular hydrogen from formate is the work of the two enzymes formic dehydrogenase and hydrogenase. Such a hypothesis would of course be invalidated if an organism could be found able to produce hydrogen from formate yet not possessing hydrogenase. So far, among the organisms investigated, we have not come across a definite exception of this sort; the following list illustrates this. In the case of *Bact. lactis aerogenes* the presence of

Organism	Ability to produce hydrogen from formate	Presence of hydrogenase
Bact. coli (Escherich)	+	+
Bact. coli (Houston 1)	+	+
Bact. coli (Houston 2)	+	+
Bact. coli (Houston 3)	+	+
Bact. acidi lactici	+*	+
Bact. lactis aerogenes	+	?
Bact. alcaligenes	−	−
Ps. pyocyanea	−*	−
Chromobact. prodigiosum	−†	−
Bac. megatherium	−*	−
Bac. subtilis	−*	−

* Pakes and Jollyman [1901].

† Pakes and Jollyman obtained a positive result with *Chromobact. prodigiosum*, but our strain, tested by ourselves, gave completely negative results.

hydrogenase is not thoroughly established, the reduction of methylene blue being slow and sometimes absent; we propose to investigate this point further.

According to the view suggested above, we should expect organisms possessing hydrogenase to liberate molecular hydrogen from other donators; two conditions at least would however have to be satisfied before this could occur. First, no hydrogen acceptors must be present since these would presumably seize the hydrogen before it was liberated; second, the r_H of the system must be such that the hydrogen would be liberated at some measurable pressure, *i.e.* $r_H \not> 3$, say. For instance, in the case of

$$\text{succinate} \rightleftharpoons \text{fumarate} + 2H,$$

the theoretical pressure of hydrogen in equilibrium with the system is of the order of 10^{-8} atmospheres.

Summary.

1. An enzyme has been found in a number of bacterial species which activates molecular hydrogen; for this the name "hydrogenase" is suggested.

2. By means of this enzyme hydrogen reduces molecular oxygen, methylene blue, nitrate and fumarate.

3. The properties of this enzyme are described, and its relation to other bacterial enzymes and also its function in the cell are discussed.

We wish to record our thanks to Dr Malcolm Dixon for very useful criticism in connection with this work, and to Sir Frederick Hopkins for his kind interest and advice.

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XXVIII. HYDROGENASE.

II. THE REDUCTION OF SULPHATE TO SULPHIDE BY MOLECULAR HYDROGEN.

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(*Received December 31st, 1930.*)

In a previous communication [Stephenson and Stickland, 1931] it was shown that many common species of bacteria possess an enzyme which activates molecular hydrogen; this enables them to reduce various hydrogen acceptors by means of hydrogen gas. It was there mentioned that the mixed culture, obtained from the River Ouse, in which this was first observed was able to reduce sulphate to sulphide by molecular hydrogen, and this reaction forms the subject of the present paper.

It was originally shown by Beijerinck [1895] that the hydrogen sulphide produced in mud arises anaerobically by the bacterial reduction of sulphates. Van Delden [1904] first obtained pure cultures of the organisms concerned (*V. desulphuricans* and *V. aestuarii*) in a medium in which sulphate was reduced to sulphide anaerobically at the expense of organic compounds (lactate and malate), the energy for development being derived from the reduction of the sulphate. Elion [1924] isolated a thermophilic organism (*V. thermodesulphuricans*) of the same type. Quite recently an exhaustive study of the metabolism of the sulphate-reducing bacteria has been made by Baars [1930]. Using several strains he investigated the products of oxidation of a large number of carbon compounds, and showed that the strains differed in the compounds which they were able to oxidise.

With regard to the reduction of sulphate by molecular hydrogen the only reference which we have been able to find is a statement by Niklewski [1914], who claims to have isolated an organism able to carry out this reaction, but gives no details.

Isolation and description of the organism.

In order to isolate the organism from the mixed culture, the technique employed by Van Delden was used; this consists in plating on broth agar containing sulphate and iron salts, the concentrations used being 0·5 % sodium sulphate and 0·005 % potassium iron sulphate. The plates were incubated anaerobically, and the colonies responsible for the production of sulphide were

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distinguished by being jet black owing to the precipitation of iron sulphide. After several repetitions a culture was obtained which gave only black colonies; the pure culture was known as Strain 182.

Like the other sulphate-reducers described in the literature this organism can be grown anaerobically on an inorganic medium containing sulphate and lactate, thus proving that it derives its energy from the oxidation of the lactate by the sulphate:

$$2CH_3.CHOH.COOH + 3H_2SO_4 = 6CO_2 + 6H_2O + 3H_2S + 254 \text{ Kg. Cals.}$$

It also resembles *V. desulphuricans* in being a small comma-shaped organism, strictly anaerobic and non-spore-forming.

It has been shown by Baars that the different species of sulphate-reducers, while agreeing in their general morphological and physiological characteristics, differ among themselves in their abilities to use various carbon compounds for the reduction of sulphate. We therefore tried to establish the identity of our Strain 182 by sowing it into a number of media containing a variety of carbon compounds and sulphate. The medium [Baars, 1930] consisted of

	%
K_2HPO_4	0·05
NH_4Cl	0·1
$CaSO_4$	0·1
$MgSO_4.7H_2O$	0·2
$FeSO_4$	Trace

with 30 millimols of carbon compound per litre, and the results were as follows:

Carbon compound	*V. desulph.*	*V. rübentsch.*	Strain 182
Glycerol	+	+	−
Mannitol		+	−
Glucose	+	+	−
Fructose	+	+	+
Galactose	−	+	?
Sucrose	−	+	−
Lactose	−	+	−
Maltose	−	+	−
Formic acid	+	+	+
Acetic ,,	−	+	−
Butyric ,,	−	+	−
Lactic ,,	+	+	+
Tartaric ,,	−	+	−
Succinic ,,	+	+	−

We thus have to conclude that Strain 182 is not identical with any organism studied by Baars.

Presence of hydrogenase in Strain 182.

The bacteria were grown on 1200 cc. of a medium containing 25 % of tryptic broth, 0·5 % sodium formate, 0·5 % sodium sulphate and 50 % of inorganic medium [Stephenson, 1930]. After about 5 days' anaerobic incubation, the cells were separated from the medium by centrifuging, washed twice

with Ringer's solution and suspended in 50 cc. of Ringer's solution. By the method already described [Stephenson and Stickland, 1931] it was proved that this strain actively reduces methylene blue by molecular hydrogen, *i.e.* contains hydrogenase.

	Reduction time (mins.)
Suspension (diluted 1 in 5) *in vacuo*	>180
,, in hydrogen	4
Boiled suspension in hydrogen	>180

The ability to use hydrogen is not a peculiarity of our strain, as it was found by the same method that *V. rübentschickii* also contains hydrogenase.

	Mins.
Reduction time *in vacuo*	>150
,, in hydrogen	17

Reduction of sulphate by molecular hydrogen.

(*a*) *Qualitative.* 1 cc. of a washed suspension of bacteria of Strain 182 was incubated at 37° for 48 hours with 1 cc. of 0·6 % sodium sulphate in phosphate buffer at p_H 7·4, in hydrogen and *in vacuo*. In the tubes containing hydrogen a strong smell of hydrogen sulphide and a strong reaction with nitroprusside were observed, and in the controls both tests were negative. By the same method the reduction of sulphate by hydrogen in presence of *V. rübentschickii* was demonstrated.

(*b*) *Quantitative.* The apparatus (Fig. 1) consisted of a bolt-headed flask of

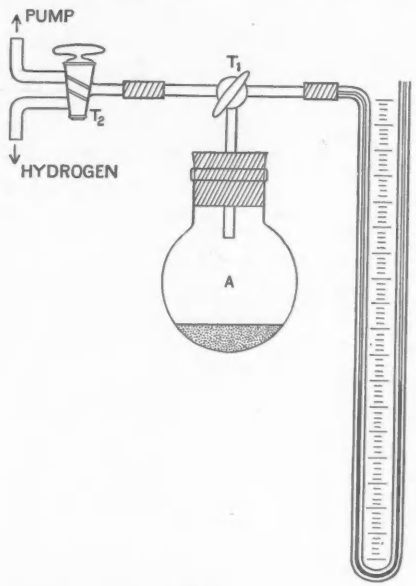

Fig. 1.

200 cc. capacity (A), fitted with a 3-way tap T_1; the latter was connected with a manometer. Into A were put 10 cc. phosphate buffer, p_H 7·2, 10 cc. of standard solution of sodium sulphate and 20 cc. of suspension of the organism. Before placing the stopper in the flask the tap T_1 was opened to the manometer, which was tipped till the mercury reached the tap; the latter was then closed by a turn through 90°. The rubber bung was then inserted in the flask, and its lower level marked on the glass. The distal tube of T_1 was then connected to a 3-way tap T_2, by which it could be joined either to a water-pump or to a supply of hydrogen. The flask was first evacuated, the liquid being shaken to expel dissolved air, and then filled with hydrogen, this process being repeated twice to ensure thorough removal of oxygen. T_2 was then closed, T_1 turned so as to connect the flask with the manometer and the flask disconnected. The whole was then placed in the incubator at 34°. When the temperature had become constant as shown by the manometer, the manometer and barometer readings were simultaneously taken, and these readings were taken at suitable intervals during the experiment until the absorption of hydrogen was complete (see Table I).

Table I.

Date (August)	Time	Manometer diff.	Uptake	Correct. for change in bar.	Uptake (corrected) mm. Hg
19	9.0 p.m.	+50·0	0·0	0·0	0·0
20	9.30 a.m.	+ 1·0	49·0	− 1·5	47·5
20	11.30 p.m.	−13·0	63·0	− 1·5	61·5
21	10.0 a.m.	−20·5	70·5	+14·5	85·0
21	10.30 p.m.	−40·0	90·0	+ 8·5	98·5
22	10.0 a.m.	−58·0	108·0	+ 1·5	109·5
22	10.30 p.m.	−63·5	113·5	− 0·5	113·0
23	9.30 a.m.	−66·0	116·0	+ 6·5	122·5
24	10.0 „	−73·0	123·0	− 0·5	122·5
25	10.0 „	−79·0	129·0	− 6·5	122·5

The stopper was then removed and the contents of the flask made alkaline by the addition of 10 cc. of N NaOH. Two 10 cc. samples were placed in 300 cc. flasks and acidified, and the hydrogen sulphide blown from them by a current of nitrogen into a series of three small absorption bottles, the first two each containing 10 cc. of $N/50$ iodine, the third about 10 cc. of toluene to absorb the iodine carried through by the current of gas. After 4 hours the iodine was titrated with $N/50$ thiosulphate, and the amount of hydrogen sulphide calculated. The residual sulphate, after removal of bacterial debris by filtration through kieselguhr, was estimated for us by a micro-method by Mr A. Colwell. The volume of the gas space in the flask and the tubes was measured.

Calculation of results.

(*a*) *Hydrogen used.* Change of pressure (corrected for change of barometer) = 445·5 mm. Hg.

Volume of gas space in flask = 134·0 cc., temperature, 34°.

$$\text{Hydrogen used} = \frac{134 \times 445{\cdot}5 \times 273}{760 \times 307} = 69{\cdot}9 \text{ cc.} = 3{\cdot}12 \text{ millimols.}$$

(*b*) *Hydrogen sulphide produced.* Iodine used by total volume = 73·7 cc. $N/50$ = 36·85 cc. $M/50$ H_2S = 0·74 millimols.

(*c*) *Sodium sulphate reduced.* The Na_2SO_4 added, 0·100 g. = 0·70 millimols, was completely reduced.

Results.

As four molecules of hydrogen are required to reduce one molecule of sulphate to sulphide, the figures for hydrogen in the table are divided by four in order to show the extent of the agreement:

$$H_2SO_4 + 4H_2 = H_2S + 4H_2O.$$

Exp.	Sulphate reduced (millimols)	Hydrogen used / 4 (millimols)	Sulphide formed (millimols)
1	0·70	0·78	0·74
2	0·40	0·38	0·35

Control experiments. (*a*) An exactly similar experiment to the above, without the addition of sulphate, showed no uptake of hydrogen. (*b*) A suspension of cells incubated with sulphate in nitrogen instead of hydrogen gave no hydrogen sulphide. (*c*) An identical experiment carried out with full sterile precautions, both in the centrifuging and washing of the organisms and in the setting up of the apparatus, gave similar results, and showed no contaminating colonies on plating at the end of the experiment.

Reduction of other hydrogen acceptors.

Baars showed that the various strains of *V. desulphuricans*, when sown into lactate synthetic medium containing sulphate, sulphite, hyposulphite, thiosulphate or sulphur, reduced them all to sulphide. We found that the washed suspension of cells of Strain 182 reduced sulphate, sulphite and thiosulphate to sulphide in the presence of hydrogen and not in the control experiments with hydrogen absent.

Effect of hydrogen ion concentration.

As hydrogenase was first studied in a group of organisms differing widely in their physiology from Strain 182, it seemed worth while to ascertain whether the enzyme showed any marked divergence of properties in association with this sulphate-reducing species. For this reason we compared the effect of hydrogen ion concentration on the enzyme of this organism with that on the enzyme in *Bact. coli* and Strain 111 previously recorded [Stephenson and Stickland, 1931]. The result is shown in Fig. 2; the explanation of the dotted lines and the lettering will be found in the earlier paper [1931, p. 210]. The curve is the same in its main character as those obtained with the other species, and it seems probable that we are dealing with the same enzyme.

The peak at p_H 6·3 gives the optimum reaction for the activation of

hydrogen; we have at present no accurate method for determining the optimum p_H for the activation of sulphate, so that the optimum for the complete reaction is unknown.

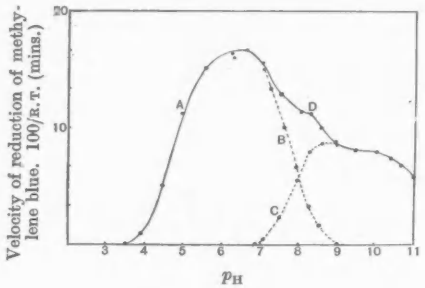

Fig. 2.

SUMMARY.

1. The enzyme hydrogenase has been found in a typical sulphate-reducing organism from the River Ouse.

2. This organism is able to reduce sulphate quantitatively to sulphide by means of molecular hydrogen according to the equation

$$H_2SO_4 + 4H_2 = H_2S + 4H_2O.$$

3. Sulphite and thiosulphate are also reduced to sulphide.

We wish to record here our thanks to Prof. A. J. Kluyver of Delft for supplying us with strains of *V. desulphuricans* and *V. rübentschickii*.

One of us (L. H. S.) is indebted to the Department of Scientific and Industrial Research for a grant.

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XXIX. THE EFFECT OF INSULIN ON GROWTH, NITROGEN EXCRETION AND RESPIRATORY METABOLISM[1].

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The clinical observation that insulin therapy in diabetes is frequently associated with a rapid increase in weight has led to considerable discussion as to whether the hormone promotes the formation of fat from carbohydrate or allows of a greater degree of sparing of fat and protein by a more complete metabolism of carbohydrate. In the diabetic subject the increase in weight is frequently brought about even when the diet is maintained at a level not necessarily in excess of what would be regarded as normal for age and weight. With the normal animal the matter is not clear. Whilst Macleod *et al.* [1930] refer to rabbits which gain weight rapidly during utilisation for the assay of insulin, Long and Bischoff [1930] could find no effect of insulin on the growth curves of these animals. The former investigators using young pigs and doses of insulin from 1·5 to 2 units per kg. could observe no very striking effect on the rate of increase in weight.

In view of these findings it is perhaps of interest to report our findings with young albino rats. In the following experiments very much larger doses of insulin were used than would *a priori* be considered compatible with the survival of the animal.

The animals were bred in the laboratory from a single pair and were of an extremely healthy and uniform strain. Comparisons were made between litter-mates only.

The method of experiment was to subject the animals of one series to a restricted diet, making certain by hand feeding that each individual received the same quantity daily. Another series was investigated with an unlimited quantity of food. Thus the appetite factor was of significance only in the second series. The diet consisted in every case of a mixture of white bread and fresh whole milk. No attempt was made to keep the diet of absolutely constant composition, but the average composition was 0·74 g. N and 17·74 g. carbohydrate (as glucose after hydrolysis) per 100 g. mixture and each animal of the restricted series received the same amount on any given day.

[1] Work performed during tenure of a Beit Memorial Research Fellowship by M. W. G.

In the case of the animals kept on an unrestricted amount of food, care was taken that the container was always well filled. The animals were all isolated in separate compartments. The doses of insulin, administered subcutaneously, varied in the animals on a restricted diet from 0·25 units to 40 units daily, always injected in one dose. In the case of the series on an unrestricted diet, the doses varied from 2 to 50 units daily, also given at one time.

The insulin used was that prepared by Messrs Burroughs Wellcome and Co.

Considerations of space allow only averages to be given, but it is to be understood that the growth curves of individual animals were practically identical.

Table I.

Litter 1. Restricted diet. Born 16. ii. 30. Weights given in g.

													% increase in wt.
Age in days	26	27	30	32	34	37	40	43	46	50	55	61	—
Controls (wt. in g.) (1 ♀, 1 ♂)	49	53·5	55·5	58	59·5	65·5	70	74	81·5	87	100	111	126
Insulinised (1 ♂, 1♀)	45·7	50	50·3	50·3	53	63	69	73	83	89	107	109	138

Litter 2. Restricted diet. Born 18. ii. 30.

Age in days	24	25	28	30	32	35	38	41	44	48	53	59	—
Controls (wt. in g.) (1 ♂, 3 ♀)	47	48	48·5	50	50	63	71	77	83·5	89	112	113	139
Insulinised (♀♀, ♂♂)	47	48	51	50	61	69	69	73	84	88	101	106	136

Insulin dosage for rats on restricted diet.

Days	0–4	5–9	10–12	13	14–18	19–20	21–24	25–26	27	28	29–35
Units per day	0·25	0·5	1·0	2	8	12	16	20	30	40	30

Litter 3. Unrestricted diet. Born 28. vi. 30.

Age in days	20	24	25	30	36	42	—
Controls (wt. in g.) (3 ♂, 3 ♀)	42	64	80	98	111	107	198
Insulinised (2 ♂, 4 ♀)	37	56	72	90	105	115	212

Litter 4. Unrestricted diet. Born 28. vi. 30.

Controls (wt. in g.) (2 ♂, 1 ♀)	38	62	82	101	114	131	249
Insulinised (1 ♂, 2 ♀)	36	60	74	95	106	123	238

Insulin dosage for rats on unrestricted diet.

Days	0–4	5–7	8	9	10–11	12–13	14–19	19–22
Units per day	0	2	4	6	10	20	40	50

Note. Of the insulinised animals, two in litter 1 and one in litter 2 were killed on the 61st and 55th days respectively by allowing them to consume all their diet in a short time and then injecting the insulin.

It will be observed from the results in Table I that the rate at which the animals put on weight was very much greater in the case of those on an unrestricted diet. Whereas the controls on the restricted diet showed an increase of about 130 % in 35 days, those on the unrestricted diet increased by over 200 % in 22 days. In this way a large range of growth activity was

obtained and any effects which insulin might have been able to produce could have been interpreted as having a broad application.

That no tolerance to large doses of insulin had developed was shown by the fatal effect of a dose of 50 units of insulin after withdrawal of food (see *Note*, Table I).

In administering such large doses of insulin, one has to consider the question of the degree to which it is eliminated in the urine. Whilst this may have accounted for a certain amount of loss, there can be little doubt that the animals were liable to a fatal hypoglycaemia during the greater part of the day, and were able to prevent its occurrence only by frequent feeding.

It is therefore not a little surprising that the hormone had, in fact, no effect whatever on either the rate of growth or on the final percentage increase in weight. Whatever theory may be held as to the essential action of insulin in the normal animal, these results appear difficult to reconcile with it. If the essential effect be regarded as a stimulus to peripheral oxidation of carbohydrate with a simultaneous drain on the glycogen of the liver, then it would have been reasonable to expect that at least in the case of the animals on the restricted diet, either a loss of weight or a diminished rate of increase in weight would have occurred. If, on the other hand, we regard the action of insulin as an inhibition of glycogenolysis, then we should have to suppose a temporary inhibition of the release of glycogen from the liver but a complete and adequate readjustment when the excess of the hormone had been eliminated. The experiments of Chaikoff and Macleod [1927] showed that rabbits on a high carbohydrate diet exhibited no increase in metabolism under the action of insulin. Lublin [1926], however, stated that, in man, simultaneous administration of carbohydrate and insulin might cause the R.Q. to exceed unity, and this he interpreted as evidence of a transformation of carbohydrate to fat. In the many experiments one of us (M. W. G.) has carried out on human subjects he has never seen such a phenomenon. Insulin indeed cannot increase the rate of metabolism and R.Q. above certain levels. When such increase cannot occur, a fall in blood sugar would only be possible if the release of carbohydrate from the liver were inhibited. Such a view is however not borne out by the fact that administration of insulin to a rat causes no increase in its hepatic glycogen, and may indeed, during active absorption, actually produce a fall.

In the present state of our knowledge it would be premature to attempt to reconcile these matters with our total inability to demonstrate a modified rate of growth by the action of insulin. Experiments on the effect of insulin on the protein metabolism of rats during starvation have demonstrated no increased protein catabolism. Since the largest dose of insulin which could be safely given was 0·5 unit it was however difficult to obtain a conclusive result. In the following experiments the plan was to subject rats to a period of 3 days' starvation, making daily collections of urine. The animals were then allowed to feed until the original weight was regained (always much more

than 3 days) after which a second period of starvation was begun during which insulin was administered. The fatalities were sufficiently frequent to make the experiment rather tedious. The urine was in most cases analysed for total N, inorganic P and creatinine.

Table II.

Day	Average initial wt. g.	Average daily excretion per 100 g. initial wt. Nitrogen g.	Inorganic P mg.	Creatinine mg.	Remarks
(*a*) Control periods of starvation. Results from 14 rats in pairs. Average:					
1	424	0·070	5·9	4·6	Starvation
2	—	0·057	6·5	4·1	,,
3	—	0·064	6·5	3·9	,,
(*b*) Periods of starvation and insulin. Results from 3 pairs from above:					
1	389	0·099	4·4	3·5	Starvation
2	—	0·107	8·3	3·1	Starvation and insulin
3	—	0·060	5·2	2·2	,,

The doses of insulin were 0·5 unit per animal daily.

On the second day one of the animals died in hypoglycaemia; exp. continued with the remaining two pairs. On the third day all the animals were hypoglycaemic; it was thought necessary to give one pair a few drops of sugar solution *per os*; hence drop in N excretion. No convulsions at any time.

Table III.

Larger doses of insulin.

Three adult rats were used and subjected to a preliminary starvation of 3 days, allowed to recover their original weight and then starved and treated with insulin.

	Initial period of starvation			Insulin period		
Day	1	2	3	1	2	3
Insulin daily (units)	—	—	—	1·5	1·0	1·0
Average N per 100 g. initial wt.	0·070	0·057	0·057	0·040	0·062	0·083

On the first day one animal died in hypoglycaemia, continued with remaining two.

We think that these results (Tables II and III) justify the conclusion that, in the starving normal rat, even in hypoglycaemic doses, insulin does not increase the catabolism of protein. When, however, as in pair 3 on the third insulin day (Table II), glucose is given, there occurs a very sharp drop in excreted nitrogen. The incidence of convulsions would of course increase the general catabolism, but in the above experiments these did not occur, so that our remarks apply to the primary effect of insulin and not to the secondary manifestations of hypoglycaemia.

In spite of the very large literature on the effects of insulin on metabolism, it cannot be said that we are clear as to its essential action. The difficulty of dissociating its effects from those of other hormones acting simultaneously in the intact animal has encouraged some workers, notably Dale and his collaborators, in the use of eviscerated and decapitated preparations. The relationship of their preparation to the living animal must however be remote. Effects can be observed to follow the injection of insulin into normal animals which

seem to be irreconcilable with the results obtained in the perfused preparation. Thus, in the latter glucose disappears from the perfusate, the oxygen utilisation rises very considerably and synthesis of glycogen occurs in the muscles, the R.Q. of the preparation remaining unity throughout. In the intact animal on a high carbohydrate diet, however, administration of insulin produces no rise in R.Q. or oxygen utilisation, whilst in the starving animal, insulin injection is followed by a rise in R.Q. and a definite rise in oxygen utilisation. Further in certain species of animal a very large rise in liver glycogen can be demonstrated to follow relatively large doses of insulin during starvation, whereas in others a definite loss occurs. The matter becomes even more confusing when we add the above findings that enormous doses of insulin do not affect the rate of growth of normal rats on restricted or unrestricted diet or their excretion of nitrogen in starvation. These latter facts, it seems to us, make it clear that the rat possesses mechanisms by means of which the tendency for insulin to increase the peripheral oxidation of sugar is effectively balanced by a diminished utilisation of other substances, probably fat. In other words, the effect of insulin is to produce a diversion of metabolism in the direction of carbohydrate. This causes a rise in R.Q. and may or may not produce an increase in total metabolism according to the readiness with which these mechanisms are brought into action. Thus, as will be demonstrated in the human subject, a rapid increase in heat production and R.Q. can occur after injection of insulin; but a progressive fall in blood-sugar may also occur without any such rise in R.Q. or metabolic rate (*v. infra*). It seems to us that this can only be explained by attributing to insulin the double action of inhibiting glycogen release from the liver and stimulating peripheral oxidation, these effects not occurring simultaneously. The rat appears normally to possess extraordinarily active mechanisms for the mobilisation of its hepatic glycogen for the needs of its peripheral tissues, so that the administration of insulin is never followed by a demonstrable rise in liver-glycogen, such as has been found both in the young cat and rabbit [Goldblatt, 1930]. It is perhaps relevant here to describe certain experiments carried out on young dogs to illustrate that the latter effect is not as unusual as the recent work of Corkill [1930] might lead us to believe.

The methods used were those described by one of us (M. W. G.) in previous communications. Comparisons were made on litter mates. The insulinised animals were always killed before any symptoms occurred. The values for muscle-glycogen were obtained from a mixed sample from all four limbs.

It is to be observed that the effect here described is by no means as striking as that found in young rabbits nor are the control values as uniform as is desirable in such comparison experiments. But it is clear that, as in young cats and rabbits, a dangerous hypoglycaemia can occur even when large amounts of glycogen are still available in the liver and before any marked change has occurred in the muscle glycogen. In so far as we are justified in taking averages of these rather divergent figures, the conclusion may be drawn

The effect of insulin on young dogs.

Animal No.	Wt. g.	Blood sugar g. per 100 cc.	Plasma inorg. P mg. per 100 cc.	Glycogen: Liver %	Glycogen: Liver Total mg.	Glycogen: Muscle %	Remarks	
				Litter 1. 24 hrs. starvation.				
1	530	0·102	8·60	0·84	140	0·82	Control	
2	510	0·104	7·93	1·47	265	0·69	,,	
3	375	0·095	7·86	2·06	258	0·44	,,	
				Av. **1·46**		Av. **0·65**		
4	576	"0"	6·46	2·21	417	0·53	11.20 a.m.	0·5 unit
							12.45 p.m.	0·5
							1.40	1·0
							2.25	Killed
5	600	0·037	—	2·22	460	0·66	11.23 a.m.	0·5
							12.45 p.m.	0·5
							1.40	1·0
							2.10	Killed
6	560	0·077	6·60	1·73	347	0·78	12.05 p.m.	0·5
							1.10	2·0
				Av. **2·05**		Av. **0·66**	1.30	Killed
				Litter 2. 48 hrs. starvation.				
1	662	0·087	7·81	0·28	56	0·59	Control	
2	790	0·090	7·86	0·79	209	0·40	,,	
				Av. **0·53**		Av. **0·49**		
3	732	0·018	6·28	0·87	216	0·55	11.30 a.m.	1 unit
							1.00 p.m.	1 ,,
							2.15	Killed
4	654	"0"	6·00	1·09	242	0·51	11.30 a.m.	1 unit
							1.00 p.m.	1 ,,
							2.00	Killed
5	417	"0"	—	0·48	72	0·60	11.30 a.m.	1 unit
				Av. **0·81**		Av. **0·55**	1.00 p.m.	1 ,,
							1.15	Killed

that an increase was occurring in liver-glycogen without any attendant decrease in peripheral glycogen. In the publication of Corkill, above referred to, the increase in the liver-glycogen of insulinised young starving rabbits was associated with an average fall in muscle-glycogen and it was calculated that the peripheral loss was at least as great as the gain in the liver. His emphasis on this matter seems to suggest that it was considered that a transference was occurring from the muscles to the liver, an assumption for which there is at present no experimental evidence. In our experiments such large peripheral losses have not been found and the increase in liver-glycogen may certainly occur when no loss at all has been sustained by the muscles.

There seems to be no question that insulin stimulates peripheral oxidation and hence we must consider that in the above experiments and in those on young rabbits and cats, either there was proceeding a simultaneous replenishment of glycogen in the muscles or the effect on oxidation is a later one. Indeed Macleod [1930] has recently suggested that this accumulation of glycogen in the liver after insulin is an early effect of the hormone; but it is

sometimes difficult to accept this because the animal may die in hypoglycaemia with an immensely increased liver-glycogen and a very considerable though diminished muscle-glycogen. In some cases a synthesis of glycogen could be demonstrated in muscle as well as in the liver [Goldblatt, 1930].

It is clear that no theory of the action of insulin can approach the truth which does not take into account that it can produce a fall in blood-sugar in the perfused decapitated eviscerated cat. But this action of the hormone seems to be influenced in the intact animal by the presence of the liver and other mechanisms, which are available to different extents in different species. Further we have no evidence as to the amount of insulin which is normally operative in different species; so that when we superimpose doses of insulin in acute experiments, the normal mechanism or its velocity is probably entirely disturbed.

In the following experiments we propose to show that even in the human subject the effect of insulin on the respiratory metabolism may vary with the conditions.

One of us (M. W. G.) was the subject of experiment. Starvation was for a period of 16 hours before the administration of the insulin. Estimations of metabolism were carried out by the Haldane and Douglas methods. The estimations of the basal values were made after 2 hours' complete rest.

Table IV. *Effect of insulin on the respiratory metabolism of a starving human subject.*

10 units insulin injected subcutaneously at 1·16.

Time	Blood-sugar g. per 100 cc.	Ventilation per min. litres	O_2 per min. cc.	CO_2 per min. cc.	Total R.Q.	Non-protein R.Q.
11.20	0·109	—	—	—	—	—
12.05	—	5·66	226	186	0·83	0·84
12.50	—	5·16	206	165	0·80	0·80
1.46	—	4·93	179	145	0·81	0·82
2.05	0·045	—	—	—	—	—
2.10	—	6·78	246	242	0·98	1·01
2.20	0·062	—	—	—	—	—
2.42	—	5·72	200	176	0·88	0·92
2.50	0·067	—	—	—	—	—

At 2 p.m. a moderately severe hypoglycaemic reaction was experienced, apprehension, sweating and hunger being marked. It will be observed that at the time of greatest fall in blood-sugar, the R.Q. had reached unity and the O_2 utilisation had increased very definitely. This increase in oxygen utilisation was the result of the greater ventilation and not of an increased difference in the compositions of the inspired and expired air. The CO_2 difference, however, rose from 3·6 to 3·9, so that it may be considered that there was a true increase in oxidation of carbohydrate with a secondary stimulus to the respiratory centre from the rise in CO_2 production. Recovery was spontaneous and the rise in blood-sugar was accompanied by a fall in R.Q. and total metabolism.

This type of metabolic change after injection of insulin is, in our experience, always reproducible in the starving organism. The apparent drop in protein metabolism is unusual and is quickly balanced by the subsequent rise. Such results give considerable support to the view that insulin provides a stimulus to the oxidation of carbohydrate, but the matter is more complicated than this.

In the following experiments (certain of which were carried out with Dr William Maclean) it will be demonstrated that:

(1) The fall in blood-sugar produced by insulin is not necessarily accompanied by a rise in R.Q.

(2) The rise in R.Q. which follows the injection of insulin in the fasting state can be inhibited by giving glucose simultaneously.

(3) The reduction of blood-sugar to negligible proportions may be accompanied by an acute fall in R.Q.

These experiments were performed on diabetic patients and on glycosurics without ketosis.

The effect of insulin on a glycosuric without ketosis.

The patient was a woman of 50 years and 15 st. weight. In Exp. A a light meal was taken at 8.30 a.m. In B starvation for 16 hours before the experiment.

	Time	Blood-sugar g. per 100 cc.	R.Q.	O_2 per min. cc.	Urine-sugar g. per 100 cc.
Exp. A	10.56	0·304	0·89	275	3·80
	11.08	10 units insulin subcutaneously			—
	11.35	0·328	0·85	286	—
	12.05	0·286	0·84	271	2·74
	1.05	0·230	0·83	255	0·41
	2.05	0·200	0·81	258	0
	2.38	0·181	0·85	252	0
	4.20	0·130	0·83	244	0
	5.30	0·117	—	—	—
Exp. B	11.25	0·348	0·74	304	3·34
	12.25	5 units insulin subcutaneously			
	12.55	0·301	0·68	296	2·00
	1.35	0·275	0·77	262	1·76
	2·15	0·240	0·72	281	0·66
	3·00	0·212	0·76	272	0·18
	4·26	0·177	0·75	261	0

The subject was a woman of active life (a washerwoman) and complained only of obesity. We were unable by any therapeutic measures to influence her obesity, partly because she did not reduce her intake of food. She was losing regularly about 100 g. sugar per day in the urine. Her threshold for sugar was definitely raised, as can readily be seen above.

It is clear from the above figures that the prevention of the glycosuria was not accompanied by any change in the degree or type of metabolism, so that we must suppose an inhibition of release of sugar from the liver to be taking place under the action of the hormone. It is important to note that this type of patient rarely shows evidence of ketosis and, in fact, in this case there was never any excretion of acetone which could be estimated. The conditions

usually taken as evidence of gluconeogenesis were absent in spite of the great loss of sugar and the high fasting blood-sugar.

It thus appears that a steady fall in blood-sugar may be induced without any observable change in metabolism. Of course the doses were small and give no information of what may occur in severe hypoglycaemia. In the next experiments very much larger amounts of insulin were used and very low levels of blood-sugar were obtained; the subjects were diabetics.

The effect of insulin on diabetic subjects.

Case 1. A young woman of 19 years. Very severe diabetic with all the classical symptoms. Always strong ketosis present. Before each experiment food was stopped for 16 hours and insulin not injected after the morning dose of the previous day.

We first give the reaction of this subject to glucose and fructose.

Time after sugar (hrs.)	Blood-sugar g. per 100 cc.	R.Q.	O_2 per min. cc.	Urine-sugar g. per 100 cc.	Urine-sugar Total during experiment g.
		Effect of 50 g. glucose *per os*.			
0	0·286	0·79	260	3·3	
½	0·390	0·71	264	6·1	
1	0·420	0·74	281	6·6	
1½	0·410	0·75	259	8·5	
2	—	0·74	248	7·1	43·31
		Effect of 50 g. fructose.			
0	0·373	0·69	227	7·25	
½	0·403	0·80	232	6·42	
1	0·423	0·68	259	7·25	
1½	0·443	0·76	233	7·62	
2	0·414	0·78	219	7·75	40·2

These results present the classical features of a very severe diabetic. A certain amount of support is given by these findings to the view that the diabetic organism can make somewhat better use of fructose than of glucose.

In the following two experiments the effect of insulin was examined.

Time after insulin (hrs.)	Blood-sugar g. per 100 cc.	R.Q.	O_2 per min. cc.	Urine-sugar g. per 100 cc.	Urine-sugar Total g.
	Effect of 50 units insulin (subcutaneously) followed 1½ hours later by 50 g. fructose.				
0	0·343	0·77	209	8·30	
0	—	0·74	235	—	
1	0·252	0·75	215	6·6	
1½	0·209	0·91	211	6·05	33·5
	50 g. fructose *per os*.				
2	0·187	0·88	227	2·80	
2½	0·206	0·85	235	1·50	
3	0·166	0·73	227	1·03	
3½	0·162	0·80	236	0·50	0·98
	Effect of 50 units insulin and 50 g. glucose *per os* at same time.				
0	0·187	0·76	258	—	
½	0·388	0·68	257	6·60	
1	0·398	0·72	241	7·10	
1½	0·353	0·76	238	8·50	
2	0·302	0·72	229	9·00	
3	0·209	0·73	237	5·30	
4½	0·104	0·76	235	0·85	34·64

The peculiar fact emerges from these findings that whereas insulin produced a rapid rise in R.Q. whilst the patient was starving, this effect was either diminished by giving fructose or completely inhibited if glucose were given simultaneously with the insulin (of course this is not specific). Further no significant change in the respiratory metabolism occurred even when the blood-sugar reached normal levels in the latter case.

Thus again it seems that insulin may promote a synthetic action without any simultaneous change in the oxidative processes.

In the following case similar observations were made.

Case 2. Patient was a girl of 14 years. Severe diabetic. Experiments were performed 16 hours after food and insulin.

Time after sugar (hrs.)	Blood-sugar g. per 100 cc.	R.Q.	O_2 per min. cc.	Urine-sugar g. per 100 cc.	Urine-sugar Total g.
		Effect of 30 g. glucose *per os*.			
0	0·420	0·69	215	5·65	
½	0·505	0·72	208	6·30	
1	0·465	0·77	201	6·82	
1½	0·408	0·83	200	7·10	
2	0·371	0·75	204	6·82	34·7
		Effect of 30 g. fructose *per os*.			
0	0·353	0·78	183	4·75	
½	0·420	0·86	186	5·12	
1	0·381	0·76	206	6·38	
1½	0·383	0·88	197	6·56	
2	0·381	0·93	189	6·38	28·0

In this case we again see some evidence that the diabetic may make a more successful use of fructose than of glucose.

The findings in this case serve to show how various may be the reactions

Time after insulin (hrs.)	Blood-sugar g. per 100 cc.	R.Q.	O_2 per min. cc.	Urine-sugar g. per 100 cc.	Urine-sugar Total g.
		Effect of 50 units insulin followed by 30 g. fructose 1½ hours later.			
0	0·388	0·78	142	7·10	
1	0·201	0·96	155	5·40	
1½	0·133	0·84	178	1·03	35·0
		30 g. fructose *per os*.			
2	0·067	0·85	162	0·30	
2½	0·022	0·77	205	0	0·30
	The very low blood-sugar reached at this time made it necessary to give some carbohydrate. 50 g. glucose were accordingly given *per os*.				
3	0·090	0·90	204	0	0
		Effect of 50 units insulin and 30 g. glucose 1½ hours later.			
0	0·374	0·75	201	5·65	
½	0·338	0·77	—	5·25	
1	0·302	0·78	204	5·77	
1½	0·333	0·79	198	6·30	30·0
		30 g. glucose *per os*.			
2	0·328	0·82	204	6·17	
2½	0·339	0·84	202	7·35	
3	0·344	0·78	192	8·17	
3½	0·296	0·76	201	7·76	14·6

of the respiratory metabolism to insulin even in the same subject. For whereas on one occasion 50 units produced an acute rise in R.Q. to 0·96 with a fall in blood-sugar from 0·388 to 0·201, on another occasion the same dosage and conditions only raised the R.Q. from 0·75 to 0·78 and the blood-sugar fell only from 0·374 to 0·302.

Very striking was the sharp drop in R.Q. (0·77) which occurred when the blood-sugar fell to 0·022. We have observed this on several occasions and can only attribute it to a retention of CO_2 in the tissues as a result of some unknown factor. There is of course the possibility that at so low a level of glycaemia there may be so great a deficiency of circulating carbohydrate that other substances are brought into the metabolic field, thus lowering the R.Q. As we have reason to believe that these substances cannot be protein, they might possibly be fat, a view which would be in harmony with the increased excretion of ketones which occurs during insulinisation of fat-fed rats.

Attention may be drawn here to the fact that it has been found that in hypoglycaemia the venous blood is highly oxygenated [Klein, 1930; Klein and Holzer, 1929] and approximates in its O_2 content to arterial blood. The arterio-venous CO_2 difference was also found by these authors to be very much diminished. They conclude that the O_2 utilisation by the tissues in insulin shock is very greatly diminished. This is, however, a conclusion which seems to us quite untenable, seeing that estimations of respiratory metabolism will almost always demonstrate a greatly increased utilisation of O_2 and especially in hypoglycaemia. If through some interference with capillary function the removal of CO_2 were interfered with, then the sudden fall in R.Q. might be accounted for. In the following experiment we show another example of the same phenomenon of fall in R.Q. in extreme hypoglycaemia.

Case 3. The patient was a mildly diabetic man. All experiments carried out after 16 hours without food or insulin.

Time after sugar (hrs.)	Blood-sugar g. per 100 cc.	R.Q.	O_2 per min. cc.	Urine-sugar g. per 100 cc.	Urine-sugar Total g.
		Effect of 50 g. glucose *per os*.			
0	0·146	0·76	208	0·20	
½	0·171	0·74	224	0·20	
1	0·206	0·95	194	0·85	
1½	0·241	0·89	185	2·15	
2	0·246	0·87	197	2·45	3·51
		Effect of 50 g. fructose.			
0	0·241	0·84	244	1·95	
½	0·261	0·87	257	1·50	
1	0·281	0·84	238	1·95	
1½	0·277	0·85	275	2·07	
2	0·271	0·85	297	2·70	15·16
		Effect of 25 units insulin and 50 g. glucose at same time.			
0	0·129	0·73	209	0·24	
½	0·146	0·72	240	0·25	
1	0·206	0·83	209	1·25	
1½	0·231	0·82	209	2·05	
2	0·222	0·83	211	1·64	
2½	0·166	0·80	204	0·95	3·54

Effect of 20 units insulin followed 1 hour later by 50 g. fructose *per os.*

Time after insulin (hrs.)	Blood-sugar g. per 100 cc.	R.Q.	O_2 per min. cc.	Urine-sugar g. per 100 cc.	Urine-sugar Total g.
0	0·156	0·74	242	0·70	
½	0·166	0·76	252	0·37	
1	0·150	0·84	239	0·27	2·80
	50 g. fructose *per os.*				
1½	0·109	0·92	254	0·25	
2	0·104	0·92	272	0·34	
2½	0·060	0·91	277	0·26	
3	"0"	0·79	271	0	0·60

Thus it is seen that the administration of glucose simultaneously with the insulin did not produce as great a change in R.Q. as when the glucose was given alone.

Again we find that very low levels of blood-sugar (in this case the symbol "0" indicates that the thiosulphate equivalent of the Cu-reducing substances in 0·2 cc. blood is less than 0·2 cc. ($N/400$)) were associated with an extraordinarily rapid fall in R.Q. not accompanied by any change in oxygen utilisation and in spite of the large amount of available sugar.

In case 2 the sudden fall of R.Q. at the blood-sugar level of 0·022 was associated with a large rise in oxygen absorption (43 cc. per min.) but the increase in CO_2 output (20 cc. per min.) was not sufficient to keep the R.Q. up. In case 3 just considered there was no rise in oxygen utilisation at the critically low blood-sugar but the CO_2 output suddenly fell. It seems to us highly improbable that this sudden change in R.Q. is due to a change in type of metabolism and we consider it much more likely that we are dealing with some retention of CO_2 not at present explicable. Such low levels of blood-sugar as we have reached have not, to our knowledge, been reported in the literature in association with estimations of respiratory metabolism and as it is not often that one dares, in the human subject, continue to such low levels, the phenomenon may be readily overlooked.

Results of such a kind raise the old difficulty which assails every attempt to interpret respiratory quotients. However, from the above results we think that the conclusion may be drawn that insulin may bring about a definite stimulus to carbohydrate oxidation but that this is not the only mechanism by which the blood-sugar is lowered, since the effect may quite certainly be produced without any perceptible change in respiratory metabolism.

A complete knowledge of the action of insulin must involve the power to foretell exactly what will occur in given conditions. It must be admitted that we cannot as yet do this. In one condition, however, *viz.* in starvation, insulin seems always to produce an increase in R.Q. but not necessarily a rise in heat production. When the conditions are made more complicated, as by administration of carbohydrate or the production of extreme hypoglycaemia, it appears that secondary influences enter which make it difficult to interpret the findings of respiratory metabolism. Where, however, in the presence of

large amounts of sugar, the R.Q. does not change after the administration of insulin in large doses, it seems reasonable to accept glycogen synthesis as the most probable explanation.

Further experiments on the effect of insulin on nitrogen excretion.

In certain of the above experiments the effect of insulin on the protein metabolism of rats in starvation was examined and it was found that no appreciable effect occurred. In the following series an attempt was made to see if the injection of the hormone during periods of exclusively fatty diets could modify the excretion of nitrogen.

Ketones were estimated by a method described by one of us [Goldblatt, 1925]. The animals were albino rats bred in the laboratory and kept previously to the experimental period on a diet of bran, oats and occasionally green stuff.

Exp. 1. An adult rat was starved during 3 days and treated as below.

		Urine		
Day	Wt. g.	Total N g. per 100 g. initial wt.	Total ketones as mg. acetone	Remarks
1	207	0·072	1·10	Starvation
2	197	0·063	0·80	Starvation
3	185	0·058	0·80	Starvation and 0·4 unit insulin at 2.30 p.m. and 4.50 p.m.

Clearly no significant change in either nitrogen or ketones occurred.

Exp. 2. This experiment was carried out during the summer, which will explain the type of ketone excretion which occurred. An adult rat was taken and put on a diet of lard and 5 % salt mixture (Wigglesworth) and water. Treatment as below.

1	163	0·047	0·80	Fatty diet commenced
2	160	0·068	3·20	
3	154	0·040	21·00	
4	152	0·042	4·50	
5	152	0·027	0·80	Adaptation complete
6	152	0·040	2·50	2 doses 0·4 unit insulin
7	158	0·046	1·10	1 dose 0·8 unit insulin

Exp. 3. The same rat and the same diet as in the last experiment.

1	—	0·039	1·00	Fat diet commenced
2	—	0·075	2·40	
3	—	0·080	17·50	
4	—	0·053	10·90	
5	—	0·045	8·60	
6	—	0·065	8·20	Adaptation almost complete
7				
8	—	0·038	3·10	2 doses 0·4 unit insulin
9	—	0·032	1·10	
10	—	0·040	0·90	2 doses 0·4 unit. Animal weak and hypoglycaemic about 2 hours after the second injection. No convulsions

On neither diet was the nitrogenous excretion modified by insulin. Excretion of ketones was increased by insulin in the rats on the fatty diet in the summer [*cf.* Burn and Ling, 1928].

In the next experiment similar estimations were made with the addition

that the period was extended, the exact amount of fat eaten per day was determined and rather greater doses of insulin were administered.

Exp. 4. Two rats were used in the same cage. The fat diet was the same as that used in the previous cases. The diet was fed by hand, so that the exact amount eaten per day was known.

			Urine		
Day	Wt. g. (combined)	Fat eaten g.	N per 100 g. animal g.	Ketones as mg. acetone	Remarks
1	287	0	0·074	1·30	Starvation
2	259	17·53	0·092	1·60	
3	266	17·95	0·061	2·00	
4	270	8·92	0·040	2·20	Ketosis of winter type
5	266	3·02	0·041	1·20	
6	254	4·97	0·048	0·80	Adaptation complete
7	245	7·32	0·047	2·10	0·28 g. $NaHCO_3$ taken by animals from a solution put in place of water
8	242	19·96	0·042	3·50	0·24 g. $NaHCO_3$
9	237	6·12	0·037	1·90	Each 3 doses of 0·3 unit insulin—no symptom
10	—	8·14	—	6·30	Each 0·5 unit—both hypoglycaemic
11	226	9·20	0·038	2·10	No treatment
12	225	7·36	0·049	3·90	Each 0·5 unit
	Total	110·49	1·549	29·00	

The total fat excreted in the faeces was <2 g.

This experiment demonstrates clearly the existence of a basal nitrogen excretion on a nitrogen-free diet, in this case about 0·04 g. N per 100 g. body weight. Further this quantity is independent of the actual amount of fat ingested daily. The insignificant excretion of ketones confirms the findings of other workers, who find that the commonly held views on ketosis are in no sense applicable to the rat.

The changes in the excretion of nitrogen after insulin were clearly of no significance and again confirm the findings in the previous experiments. It will also be seen that the excretion of ketones was, after adaptation was complete, increased to a greater extent by insulin than by $NaHCO_3$ administered under similar conditions. These sudden rises in ketosis after insulin are not readily explicable. The view taken by Hirschhorn and Pollak [1927] as to the action of adrenaline on ketosis may be partly applicable to that of insulin, *viz.* that there occurs a sudden drain on the hepatic glycogen and that it is the suddenness of this drain which determines the degree of increase of ketosis. On this view a quantitatively equal loss of glycogen more gradually released would be followed by a smaller degree of ketosis.

The interesting fact that rats can continue for so long as in the above experiments in health and relatively slow rate of loss in weight on a diet consisting solely of fat and salts raises the contested question as to whether fat can give rise to carbohydrate. There can be no doubt that rats killed after prolonged periods of exclusively fatty feeding show very considerable quantities of glycogen in the liver and tissues. This matter will however be dealt with in a further communication.

Conclusions.

From these experiments it appears justifiable to consider that, in the normal animal, starving or consuming a diet containing no nitrogen, there is no change in the excretion of nitrogen as a result of the injection of insulin. The effect of insulin therefore appears to be limited to the metabolism of fat or carbohydrate or both.

The effect of insulin on peripheral oxidation is not one which, as far as our evidence goes, can exert any profound influence on the general total metabolism, as was seen in our growth experiments with rats. Nor is an increase in oxidative processes a necessary accompaniment of the fall in blood-sugar which follows the injection of the hormone.

Summary.

1. Litters of white rats, kept on restricted or unrestricted amounts of food, showed no difference in rate of growth from that of litter mates, when injected with daily doses of insulin varying from 2 to 50 units.

2. Insulin was found to have no effect on the nitrogen excretion in the urine of rats consuming an exclusively fatty diet or during starvation. This was the case whether hypoglycaemia did or did not occur; convulsions were absent.

3. Insulin hypoglycaemia in a starving human subject is associated with a marked increase in R.Q. and hence presumably an increased carbohydrate- and diminished fat-oxidation. This effect passes over as the blood-sugar recovers.

4. Progressive fall in blood-sugar and disappearance of glycosuria may be induced in a glycosuric patient without any change in R.Q. or calorie output. It is considered that this must indicate glycogen synthesis in the liver.

5. Insulin can increase the liver-glycogen of young dogs during starvation without any change in muscle-glycogen. This effect may be accompanied by profound hypoglycaemia.

6. The attainment of blood-sugar values of vanishing proportions may be accompanied by an acute fall in R.Q. It is considered improbable that this fall indicates a true change in metabolism.

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XXX. THE DISTRIBUTION OF NITROGEN AND SULPHUR IN THE URINE DURING CONDITIONS OF INCREASED CATABOLISM.

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As the result of a series of analyses of the urine of normal subjects on standard diets of varying nitrogen and sulphur content but practically free of purine, creatine and creatinine, Folin [1905] advanced the hypothesis that there are two essentially different forms of protein catabolism, the constant or endogenous catabolism yielding chiefly creatinine and neutral sulphur and to a less extent uric acid and ethereal sulphates, and the variable catabolism yielding chiefly urea and inorganic sulphates. Folin stated that "the more the total catabolism is reduced the more prominent become these representatives of the constant catabolism, and the less prominent become the two representatives of the variable catabolism."

Whilst Folin's general hypothesis has been accepted, doubt has been expressed as to whether these various excretory products can be exactly divided into two such groups.

More recent work has demonstrated the difficulty of correlating the creatinine output with the total tissue or endogenous metabolism. Shaffer [1907; 1908, 2], Spriggs [1907, 1, 2], Paton [1910] and Benedict and Osterberg [1923] consider that creatinine is an index of only one phase of this tissue metabolism —that which is peculiarly related to muscle. Spriggs also concluded from his observations that the bulk of the endogenous creatinine output is "a product of the internal structural metabolism of muscle and not of its contraction." Some 30 years ago Sivén [1901] and Burian and Schur [1901] showed severally that uric acid is to a large extent independent of the total amount of nitrogen eliminated. Folin states that while uric acid does not exhibit the quantitative constancy that is found in the case of creatinine at least a certain part of it represents the breaking down of living protoplasm which must be supposed to be essential to the continuation of life. The reduction in the uric acid output when the total metabolism of protein is reduced is irregular and different for different individuals. Pohl [1917] has more recently suggested that uric acid might be included as a principal endogenous catabolite.

Practically all experiments bearing on this aspect of metabolism have been concerned with studying the effect of different levels of food intake on the partition of the nitrogen- and sulphur-containing catabolites of the urine. Starvation may be taken as an extreme example of this type of experiment. Benedict [1907] found that preformed creatinine and neutral sulphur decreased persistently during fasting. The interesting point arises however as to the effect of increased catabolism on the distribution of the nitrogen and sulphur of the urine where the food intake is kept constant. The main causes generally accepted for such increased protein catabolism are increased muscular work, disuse of muscle, injuries to and toxic conditions of the tissues including febrile states, exophthalmic goitre and various other wasting diseases.

Gregor [1900] concluded that hard physical exercise produces a decided increase in creatinine elimination particularly on the day following the exercise. Campbell and Webster [1921, 1922] and Rakestraw [1921] have also noted an increased output.

While Shaffer [1908, 1] found that muscular work had no influence on the excretion of uric acid, Kocher [1914], Rakestraw [1921] and Levine *et al.* [1924], have since found that it caused a slight increase. Others like Dunlop *et al.* [1897], Campbell and Webster [1921, 1922] and Hartmann [1924] however record a decrease.

While Shaffer [1908, 1] and Campbell and Webster [1921, 1922] have found that work exercises but little influence on the output of ammonia, Dunlop *et al.* [1897] have observed a rise, and Kocher [1914] a definite reduction (nitrogen-free diet) in the output of this catabolite.

In more recent carefully controlled experiments on the human subject, Cathcart and Burnett [1926] have detected a slight but constant rise in the urinary excretion of nitrogen and sulphur after work. This was accompanied by a slight uniform rise in the excretion of ammonia, creatinine and urea, although the latter was not directly analysed. Dunlop *et al.* [1897], Shaffer [1908, 1], Kocher [1914] and Campbell and Webster [1921, 1922] have also found that for the most part the definite rise in the nitrogen after work is in the form of urea.

The present writer [Cuthbertson, 1929], found that subjects in nitrogenous equilibrium showed within a day or two from the commencement of a period of rest a rise in the excretion of sulphur, nitrogen, phosphorus and calcium. The S:N ratio suggested a sulphur-rich source of the excreted material. It was found that the rise in the excretion of sulphur was due to a slightly greater proportionate increase in the inorganic sulphate fraction. Ethereal sulphates tended to decrease; neutral sulphur remained more constant. The rise in the excretion of total nitrogen was mainly due to a proportionate increase in the amount of urea. Ammonia tended also to rise proportionately while the absolute amounts of creatinine and uric acid remained practically unaltered. A very slight rise in the excretion of creatinine was sometimes noted, particularly as the period of enforced rest lengthened.

In various febrile conditions, it has been found that on the average 82 % of the total nitrogen in the urine is in the form of urea [von Noorden, 1907]. Ammonia excretion is also increased considerably, but the percentage increase is generally not greater than the percentage increase in the total nitrogen. The endogenous output of uric acid is also increased, the increase coinciding and terminating with the fever [Cathcart, Kennaway and Leathes, 1908]. Although the output of creatinine is almost always increased in febrile conditions the rise is not proportional to the increased elimination of total nitrogen [Leathes, 1906; Shaffer, 1908, 2]. Leathes [1906] for example found that while there was a 50 % increase in the total nitrogen, there was only a 20 % increase in the creatinine nitrogen in one particular case. In the case of uric acid the proportion excreted was considerably increased.

Sandiford *et al.* [1926] found that the injection of thyroxine on the 30th day of a protein-free diet in the human subject produced a subsequent rise in the excretion of nitrogen mainly in the form of urea, creatinine and uric acid remaining constant. Shaffer [1907 and 1908, 2] had many years ago noted that the amount of creatinine excreted in exophthalmic goitre was very low, despite the increased tissue catabolism.

The purpose of the short foregoing review has been to demonstrate that in conditions where the nitrogen and sulphur reserves of the body are being catabolised to any great extent and where there is little or no change in the food intake the main increase in the urinary excretion is accounted for as urea. The proportion of this catabolite eliminated may even be slightly greater. Creatinine on the other hand exhibits practically no change in the absolute amount excreted. Occasionally when the increased catabolism is at a high level this substance may be excreted in slightly greater amount, but it is never increased proportionately. The excretion of uric acid, while not so constant as that of creatinine, is relatively stable, such fluctuations as occur are usually in the form of a rise. It may be that there is a special metabolism of nucleoprotein. Ammonia generally tends to rise with the increased catabolic level.

Concerning sulphur not so much is known. It may be generally stated that a rise in total sulphur is due to a practically proportionate increase in the inorganic sulphates. Neutral sulphur remains practically constant. The ethereal sulphates may diminish.

Such findings as have been described appear in agreement with the laws governing the composition of the urine as enunciated by Folin [1905].

More recently the present author [Cuthbertson, 1930] has investigated, in part, the effect of tissue injury on metabolism. It was found that the urinary excretion of sulphur, nitrogen and phosphorus rose rapidly to a maximum generally within 3 to 6 days from the time of injury. The S:N ratio suggested that some sulphur-rich tissue, such as muscle, might be the main source of the material catabolised. The partitions of the nitrogen and sulphur were not determined at that time. They are amplified in the present investigation.

Table I. *The partition of nitrogen and sulphur in the urine of patients at rest in bed during their recovery from fractures of bone or minor operations.*

Case	Total daily excretion of nitrogen g.	Daily excretion and (partition) of nitrogen: Urea	Ammonia	Amino-acid	Total creatinine	Preformed creatinine	Uric acid	Total daily intake of N g.	Daily excretion and (partition) of sulphur (SO_3): Total	Inorganic	Ethereal	Neutral	Remarks
1	32·06*	26·09 (81·4)	1·56 (4·9)	1·14 (3·5)	1·29 (4·0)	—	0·195 (0·6)	11·70	4·96	4·12 (83·1)	0·24 (4·8)	0·60 (12·1)	Fourth day following a fracture of both bones of one leg of a boy aged 16 years. Average daily output of faecal nitrogen 1·51 g.
2	9·29	7·69 (82·8)	0·20 (2·2)	0·06 (0·7)	0·51 (5·5)	0·45 (4·8)	0·18 (1·9)	9·32	1·73	1·255 (72·5)	0·155 (9·0)	0·32 (18·5)	Prior to operative removal of torn cartilage from knee joint of man.
	21·31*	17·23 (80·9)	1·12 (5·2)	0·315 (1·5)	0·52 (2·4)	0·33 (1·5)	0·15 (0·70)	9·32	3·66	3·04 (83·1)	0·26 (7·1)	0·36 (9·8)	Third day after operation
3	7·66	6·41 (83·7)	0·35 (4·6)	0·04 (0·5)	0·40 (5·2)	0·39 (5·1)	0·09 (1·2)	7·06	1·79	1·34 (74·9)	0·09 (5·0)	0·36 (20·1)	Fourth day after fracture of both bones of one leg of a man.
	12·70*	11·44 (81·0)	0·28 (2·2)	0·03 (0·2)	0·42 (3·3)	0·41 (3·2)	0·18 (1·4)	7·06	2·30	1·79 (77·8)	0·14 (6·1)	0·37 (16·1)	Seventh day after
4	6·83	5·63 (82·5)	0·42 (6·1)	0·06 (0·9)	0·50 (7·3)	0·50 (7·3)	0·13 (1·9)	7·78	1·66	0·915 (55·2)	0·175 (10·5)	0·57 (34·2)	Prior to operative removal of torn cartilage from knee joint of man.
	16·06*	11·96 (74·5)	0·72 (4·5)	0·09 (0·6)	0·50 (3·1)	0·47 (2·9)	0·22 (1·3)	7·78	2·51	1·85 (73·7)	0·16 (6·4)	0·50 (19·9)	Third day after operation
5	16·90*	13·49 (79·8)	0·495 (2·9)	—	0·47 (2·8)	0·475 (2·5)	0·14 (0·83)	8·36	2·57	2·16 (84·05)	0·17 (6·6)	0·24 (9·35)	Eighth day after fracture of the femur of a man
6	10·08	8·51 (84·4)	0·515 (5·1)	0·03 (0·3)	0·58 (5·7)	0·55 (5·4)	0·10 (1·0)	12·67	2·14	1·65 (77·1)	—	—	Third day after a dislocation of the ankle of a man.
	19·74*	17·24 (87·3)	0·45 (2·3)	0·22 (1·1)	0·70 (3·5)	0·70 (3·5)	0·23 (1·2)	12·67	3·29	2·85 (86·6)	—	—	Sixth day after
7	13·33	9·85 (73·8)	0·90 (6·8)	0·16 (1·2)	0·57 (4·3)	0·57 (4·3)	0·17 (1·3)	10·77	2·79	2·23 (80·0)	0·15 (5·3)	0·41 (14·7)	Third day after fracture of tibia of a man.
	20·58*	18·45 (89·6)	0·66 (3·2)	0·31 (1·5)	0·68 (3·3)	0·625 (3·0)	0·26 (1·2)	10·77	3·81	3·07 (80·6)	0·19 (5·0)	0·55 (14·4)	Sixth day after
8	11·86	8·73 (73·6)	0·51 (4·3)	—	0·41 (3·5)	0·40 (3·4)	0·23 (1·9)	12·10	2·06	1·54 (74·7)	0·15 (7·3)	0·37 (18·0)	Prior to bone-grafting operation for ununited fracture of humerus of 6 years standing in a man.
	17·53*	15·05 (85·8)	0·91 (5·2)	—	0·63 (3·7)	0·36 (2·0)	0·25 (1·5)	12·10	2·97	2·55 (85·9)	0·09 (3·0)	0·33 (11·1)	Fourth day after operation

* Maximum daily excretion of nitrogen.

The excretion of urinary nitrogen was determined daily throughout the periods of observation, but these values are omitted except on the days on which the distribution was detailed.

Methods. Total N, Kjeldahl; urea, urease; ammonia, aeration; creatine and creatinine, Folin; amino-acids, Sørensen; uric acid, Hopkins; total sulphur, Denis; inorganic and ethereal sulphates, Folin.

The figures for excretion are g.; those for partition %.

Experimental.

The subjects were patients at rest in bed during recovery from fractures of bone or minor operations and generally within 1 or 2 days following the injury were put on a constant intake of food, the quality and quantity of which they chose from the following list: milk, brown and white bread, digestive biscuits, butter, eggs (with the exception of case 5), tea (2 teaspoonsfull infused daily: no tea in cases 5, 7 and 8), steak (except in cases 3, 4, 5, 7 and 8), potatoes, lettuce, apple and orange. As they had been on a normal diet prior to admission, it was thought that the nitrogenous intake would be closer to this normal if a meat-containing diet was consumed. Subsequently meat-free diets were selected in order to assess the endogenous metabolism. The analytical results are summarised in Table I.

Nitrogen.

Total nitrogen. The maximum total daily output of urinary nitrogen of this series occurs between the 3rd and 8th days following the injury. There does not appear to be any definite relationship between the time interval for the urinary excretion of nitrogen to reach a maximum and the total amount passed on that particular day. The maximum daily loss may even exceed 20 g. (Case 1).

Urea. There is an absolute increase in the amount of urea, which increase is practically proportional to the raised urinary output of total nitrogen (81–82 %).

Ammonia. The absolute amount of ammonia tends to vary but there is generally a relative diminution in the proportion of the total nitrogen excreted in this form.

Amino-acids. There is generally a rise in the absolute amount passed (as measured by the nitrogen content) and this rise is practically proportional to the rise in total nitrogen.

Creatinine (preformed). Except for slight increases in cases 6 and 7 there is little or no alteration in the absolute amount passed.

Uric acid. There is generally an absolute rise in the excretion of uric acid, but this rise may or may not be proportional to the increased output of total nitrogen.

Creatine. A creatinuria with a normal (or even supernormal) output of creatinine has been noted following amputations of limbs [Bürger, 1919; Cameron and Gibson, 1922], and also sometimes after fractures, particularly of the thigh or leg bones. Hirst and Imrie [1928] found that in such fractures the amount varied from traces to over 500 mg. daily. It disappeared gradually during the healing. Administration of thyroid increased the output threefold.

In cases 2, 4 and 8 only was a pre-injury period available. Case 2 was on a meat diet and cases 4 and 8 on a non-meat diet. It is seen that whereas in cases 2 and 4 the total creatinine was constant during the pre-operative

and post-operative periods there was in case 2 in the post-operative period of maximum catabolism a slight increase in the creatine fraction and in case 4 there was only a trace of creatine on the 3rd day following the operation. Prior to operation case 8 had no creatinuria, but on the day after operation there was a very definite amount present, the creatinine fraction being slightly diminished. Case 3, who was also on a meat-free diet, showed no change in the total creatinine excretion nor was any creatine present. No creatinuria was found at the height of catabolism in case 6 although on the 3rd day after admission there was a trace present. This case had a dislocated ankle but there was no fracture or surface wound present. In case 7 no creatine was present shortly after admission but on the day of the appearance of the maximum excretion of nitrogen a trace was found. It appears then that a temporary creatinuria may follow injury. It may be that this is due to an interference with the normal transformation of creatine into creatinine, such as appears to occur in conditions of heightened catabolism.

Proteinuria. On the 3rd day following injury in case 1 there was a faint trace of coagulable protein; on the 4th day there was a definite trace (day of appearance of maximum nitrogen excretion); on the 5th day a faint trace, on the 6th day a very faint trace, thereafter it was absent. In case 4 a trace was noted on the 2nd day following operation only. No evidence of a proteinuria was noted in the other cases described here, although unsystematised testing had often revealed faint traces in cases of recent fracture admitted to hospital.

Sulphur.

In a previous investigation on the disturbance of metabolism produced by injury, it was found that the maximum daily output of sulphur practically always ran parallel with that of nitrogen. As the result of this finding day-to-day determinations of the total nitrogen only were performed and when the maximum excretion was noted the partitions both of nitrogen and of sulphur were determined on that day and also on an earlier date, *i.e.* prior to the appearance of the obvious increase in catabolism. A marked increase in the excretion of sulphur was again noted to follow injury.

Inorganic sulphate. Not only is there an absolute increase in the amount of inorganic sulphate excreted but the percentage of sulphur excreted in this form is greater.

Ethereal sulphate. While there is generally a rise in the excretion of this fraction during periods of increased catabolism, this rise is not proportional to the increase in catabolism as measured by the increase in total sulphur.

Neutral sulphur. There is not quite the same constancy about this fraction as there is about creatinine, but in only one case (7) was there really a pronounced alteration in the amount eliminated as the total sulphur excretion increased. It is of interest that it was in this particular case that a marked increase in the creatinine excretion was noted. As a consequence of this

relative constancy there is a decrease, during periods of heightened catabolism, of that fraction of the total sulphur represented by neutral sulphur.

Discussion.

While these experiments are totally different in kind from those devised by Folin, yet they have an intimate bearing on the two conceptions of metabolism which he describes. It is obvious in the first place that the source of these nitrogen and sulphur losses cannot be the food intake. They must come from the increased catabolic changes in the tissue cells and body fluids. It must also be remembered that it is really only one phase of the total catabolism which has been examined, namely that particularly related to protein.

As the result of the foregoing analyses, there appear to be two distinct forms of metabolism, one constant the other variable. The constant element may exhibit small fluctuations, for example, it may increase with any general increase in catabolism. It is obvious that whilst creatinine and neutral sulphur do chiefly represent the constant catabolism they are not the main representatives of the endogenous catabolism. Urea and inorganic sulphate are the chief products of the increased endogenous catabolism. It seems necessary therefore to dissociate Folin's terms "constant" and "endogenous", "variable" and "exogenous" when describing these and other allied conditions of increased catabolism, for fluctuations in both endogenous and exogenous metabolism are mainly characterised by fluctuations in the variable group of catabolites.

The work of Morpurgo [1897], Greene [1919], Mandel [1928] and others suggests that muscle has a very considerable capacity either for storage or depletion of protein without seriously damaging the life of the individual cell.

It is suggested here that the disintegration and integration of muscle cells through wear and tear and indeed of the body generally, may be peculiarly represented by creatinine and perhaps by neutral sulphur. This does not mean that they are the sole nitrogen- and sulphur-containing catabolites of this form of metabolism. Fluctuations in the urea and inorganic sulphate fractions, although they represent mainly variations in the improvement or impoverishment quotas, may also be an index of changes in the processes of wear and tear. Ammonia is rather an index of the acid-base balance and as such is subject to pronounced variations. Alterations in the excretion of amino-acids and ethereal sulphates are difficult to interpret. Uric acid, although it appears to fluctuate to some extent with the general increase in the total nitrogen excretion, may also be taken to be an index of wear and tear. Possibly it is a catabolic product of the nuclear part of the cell.

The question arises as to the cause or causes of these various catabolic phenomena. Wasting of tissue, particularly of muscle, may be due to an inadequate supply of normal stimuli, or to an excessive number of abnormal stimuli. It may be due to direct poisoning of the tissue cells such as might be supposed to take place in febrile conditions and in tissue injury. The wasting

on the other hand may represent the response to the demand for a non-nitrogenous residue for energy purposes, or it may be part of the mechanism for the rapid production of repair material to heal an injured part. In the present state of our knowledge it is impossible to define the relative significance of these processes.

Summary.

1. The present series of experiments provides additional evidence of the early catabolic loss of nitrogen and sulphur which results from tissue injury. The rate of loss reached a maximum about the 3rd to 8th day following the injury. The maximum daily loss of nitrogen might even exceed 20 g.

2. The partition of the nitrogen- and sulphur-containing catabolites demonstrated that the increase in nitrogen was due to a practically proportionate increase in the urea excreted, and that the increase in sulphur was due to a slightly greater proportionate increase in the excretion of inorganic sulphates.

While the excretion of ammonia fluctuated, that of amino-acids and uric acid generally rose. Ethereal sulphate tended to diminish slightly. Creatinine and neutral sulphur remained practically constant, any increase being in no way proportional to the total increase of nitrogen or sulphur respectively.

3. Definite traces of creatine were observed particularly during phases of increased catabolism.

4. Traces of heat-coagulable protein were occasionally found, particularly during the time of the maximum excretion of the nitrogenous catabolites.

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XXXI. SELECTIVE FERMENTATION.

ALCOHOLIC FERMENTATION OF MIXTURES OF GLUCOSE AND FRUCTOSE BY BREWER'S AND SAUTERNE YEASTS.

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In recent years there has been a revival of interest in the phenomena of selective fermentation of mixtures of glucose and fructose by brewer's and other yeasts. Contributions have appeared from Willstätter and Sobotka [1922], Fernbach, Schoen and Mori [1927], Ivecovich [1930], Hopkins [1928], and Sobotka and Reiner [1930, 2]. Convenient summaries of the known phenomena appear in each of the last two papers. Broadly speaking, the facts calling for explanation are as follows:

(1) that glucose and fructose in separate solutions are fermented at approximately equal rates by living yeasts;

(2) that in mixtures of the two sugars, glucose is fermented faster than fructose by brewer's and most yeasts, but the reverse is the case with certain Sauterne yeasts; the selectivity was expressed by Hopkins [1928] by the formula

$$K_{G/F} = \frac{\ln a_g - \ln (a_g - x_g)}{\ln a_f - \ln (a_f - x_f)}$$

where a_g and a_f are the initial concentrations of glucose and fructose respectively, and x_g and x_f the concentrations fermented in a given time;

(3) that fructose alone is specifically esterified at least as rapidly as glucose by yeast juice or zymin prepared from brewer's yeast, and more rapidly in the presence of suitable concentrations of inorganic phosphates.

From a consideration of facts (1) and (2), it must be concluded that the total rate of fermentation is controlled by one of the reactions succeeding the stage at which selection occurs. The later reactions are apparently the same for both hexoses. The fact of selective fermentation, indeed, strongly supports the idea that the first reaction of fermentation in the living yeast cell is more rapid than a subsequent one. This is in agreement with the results of Harden and Young's work with yeast juice. Since such a majority of yeasts prefer glucose, Sauterne yeasts should be regarded as the exception. Neglecting this for the present, and considering together facts (2) and (3), the anomaly seems to be presented by brewer's and most other yeasts, and the author's attention was firstly directed to this.

It was thought possible that the rate of diffusion of one sugar into the

cell may be influenced by reaction products of the other sugar or even that one sugar may be produced from the other within the cell. For example, Harden and Young [1910] found that fructose is produced from hexose-diphosphate by the action of an enzyme, hexosephosphatase, in yeast juice. If such fructose is produced within the living cell, it would be produced at a definite rate per cell. More than one explanation of the selective action of brewer's yeast could be advanced on this hypothesis. The production of one sugar from the other *via* hexosephosphate at a fixed rate per cell should be independent of the concentrations of glucose and fructose in the external medium over wide range, so that the value of K should vary if widely differing initial proportions of glucose and fructose are used. This, however, was found not to be the case (Exp. 1).

It was shown by Kuhn and Münch [1925] that saccharase is of two types, that of brewer's yeast for example which is inhibited by fructose, and that of *e.g. Aspergillus oryzae* which is inhibited by glucose. Weidenhagen [1930] terms these two types respectively β-fructosidase and α-glucosidase. The possibility arises that the saccharases of brewer's and Sauterne yeasts are respectively of these two types, and in some way function to inhibit the fermentation of the respective sugars with which they may be assumed to combine. Investigation gave the following results.

(1) The saccharases of both brewer's and Sauterne yeasts were inhibited about twice as much by fructose as by glucose (Exp. 2). This was referred to by the author in a previous paper [Hopkins, 1930].

(2) Brewer's yeast, treated to reduce its saccharase activity relatively to its fermentative power by the method of Willstätter, Lowry and Schneider [1925], exhibited much the same selective power as before (Exp. 3). Sobotka and Reiner [1930, 2] have recently reported a similar result.

(3) Zymin prepared from brewer's yeast ferments glucose faster than fructose in a mixture, and this preference was undiminished by the addition of saccharase in the form either of a crude yeast autolysate (too old to contain any co-zymase) or of purified saccharase (Exp. 4). It should be noted, however, that the selective preference for glucose exhibited by zymin may be due simply to the production of fructose by hexosephosphatase.

The rate of fermentation of glucose and fructose in separate solutions. The approximate equality of the rates of fermentation in separate solutions by living yeast has been proved by experiments in which the reactions were of the zero-molecular type, *viz.* the rates measured were functions of the concentration of yeast and of one of the later reactions of the fermentation. Unless the sugar concentration is low enough to introduce a new limiting factor, no difference between the rates for the two separate sugars is likely to be found. It was found, however, that:

(1) Brewer's yeast ferments glucose faster than fructose when the concentration of sugar in the fermenting mixture is well below 1 %, whereas approximate equality in rate was observed above 1 % concentration.

(2) Sauterne yeast ferments glucose more slowly than fructose at any concentration up to 10 % (the maximum tested), but more decidedly so as the concentration falls (Exp. 5).

Influence of temperature on the value of $K_{G/F}$. It was observed by Hopkins [1928] that the selectivity of brewer's yeast was greater as temperature was lower. This has been confirmed, and values of $K_{G/F}$, 2·59 at 8°; 2·48 at 20°; 2·30 at 31° were obtained. Sauterne yeast yielded corresponding values for $K_{G/F}$, 0·051, 0·071, 0·098 at these temperatures respectively. The values of K were compared as far as possible at corresponding stages of the fermentations (Exp. 6). These results led to the hypothesis that brewer's yeast exerts a selective preference for that constituent of fructose which increases in proportion to rise in temperature. It is well known that the specific rotation of fructose becomes less negative by 0·64° per degree rise in temperature. This is explained by a change in the relative proportions of the constituent forms of fructose.

The mutarotation of fructose and glucose when partly fermented by the yeasts. Fructose, in accordance with the above hypothesis, mutarotated in the positive direction when brewer's yeast was used, and in the negative direction when Sauterne yeast was used. Glucose when partially fermented by brewer's yeast, showed a mutarotation in the positive direction, the value of $[\alpha]_D^{20°}$ being + 49°. Willstätter and Sobotka [1922] obtained a similar result with a Munich brewery yeast. Again, Sauterne yeast acted in the converse manner, the glucose possessing $[\alpha]_D^{20°}$ + 54° at interruption.

In any explanation of the general phenomena of selective fermentation these results must be taken into account. The simplest inference to be drawn from the mutarotations of glucose is that brewer's yeast selectively prefers the α-form ($[\alpha]_D$ + 110°) and Sauterne yeast the β-form ($[\alpha]_D$ + 19°) of glucose. On this assumption the values of $K_{\alpha/\beta}$ for the brewer's yeast are 1·4 to 1·5 at the stage of 30 to 40 % of total glucose fermented. Willstätter and Sobotka's result for the corresponding stage of fermentation was only slightly higher. The selective action of the yeasts on fructose constituents is more significant. It may be that only α- and β-forms of normal fructose exist in an aqueous solution. β-Fructose has a specific rotation of − 130° [Nelson and Beegle, 1919], the other form has not been isolated, but is presumably present only in small proportion at low temperatures, but increasingly with rise of temperature. However, the striking change in optical rotation of fructose with change of temperature, a phenomenon not paralleled in glucose or the other familiar sugars, strongly suggests that the unidentified form is one the formation of which from normal fructose involves a not inconsiderable energy change. Brown and Pickering [1897] found that 1 g. of glucose (α) evolves from 0·7 to 0·9 calorie in mutarotating to equilibrium, whereas 1 g. of fructose absorbs from 4·6 to 5·4 calories (probably only about 0·1 g. of fructose actually changes to the γ-form). Normal fructose, according to the now generally accepted formula of Haworth consists of an amylene oxide or δ-ring (pyranose).

The other form may be a butylene oxide or γ-ring (furanose). The latter probably possesses a + rotation as would be expected from considerations of the specific rotations of the tetramethyl δ- and γ-fructoses. The application of Hudson's lactone rule to γ-fructose would also indicate a + rotation. If this is so this form of fructose, if present, would be present in very low proportion in aqueous solutions of fructose at ordinary temperatures. (It should be noted that Morgan and Robison [1928] found that the hexose of hexosediphosphate is γ-fructose.)

The hypothesis is now advanced that brewer's and presumably most yeasts are specific for the normal aldo-hexoses, glucose and mannose, and for this unidentified, but probably γ-form of fructose. If, as seems probable, this latter is present only in relatively small proportion in fructose solutions, it would necessarily be taken up slowly in a selective fermentation of a mixture of glucose and fructose. Conversely, Sauterne yeasts are specific for normal fructose (or β-fructose on the simple theory of only α- and β-forms) and ferment glucose specifically more slowly than normal fructose. If this hypothesis should prove correct, the problem of the selective fermentation of these sugars by various yeasts resolves itself into a more normal one, *viz.* that brewer's and most yeasts are specific for certain hexoses, those derived directly from maltose, sucrose, *etc.*, and Sauterne yeasts for normal fructose as it occurs in grapes.

Experimental.

The glucose and fructose used in these experiments were found to give $[\alpha]_D^{20^\circ} + 52{\cdot}6^\circ$ and $- 93{\cdot}0^\circ$ respectively. The brewer's yeasts were all top fermentation yeasts obtained from Scottish breweries. Exps. 5 to 8 (Tables IV, V, VI and I, Nos. 11–20) were performed within a few weeks of each other with yeast obtained from the same source. The Sauterne yeast used in the same series was isolated from a bottle of Sauterne wine (1928), from which the yeast used in Exp. 2 had been previously isolated in 1928. It was grown in a medium of grape must and fructose.

Exp. 1. *Selective fermentations by brewer's yeast with varying initial proportions of glucose and fructose.*

Fermentations with yeasts A and B were performed at 25°, and samples withdrawn at intervals, centrifuged and filtered. The polarimetric readings were taken at 20° after allowing time for the rotations to stabilise at that temperature, and the reducing powers by the method of Lane and Eynon. The results are given in Table I (1–7).

Exp. 2. *Inhibition of the saccharase of brewer's and Sauterne yeasts by glucose and fructose respectively.*

The inversions were performed at 20° and all polarimetric readings were made at the same temperature which was never allowed to change.

Table I. *Selective fermentation of glucose and fructose by various types of yeast and under various conditions.*

		Yeast and temp.	Time (hours)	% fermented of total sugars	Sugars unfermented		$K_{G/F}$
					Glucose	Fructose	
Exp. 1.	1	Brewer's A 25°	—	0	10·06	2·45	—
				39	5·79	1·88	2·08
				63	3·25	1·41	2·03
	2	,,	—	0	6·30	5·98	—
				54	1·99	3·56	2·22
				71	0·96	2·61	2·26
	3	,,	—	0	2·64	10·00	—
				77	0·24	2·66	1·79
	4	Brewer's B 25°	—	0	9·98	1·11	—
				80	1·725	0·465	2·02
	5	,,	—	0	9·00	1·84	—
				77	1·624	0·816	2·10
	6	,,	—	0	5·04	5·16	—
				23	3·56	4·36	2·07
				77	0·833	2·22	2·13
	7	,,	—	0	1·50	8·40	—
				31	0·721	6·15	2·30
Exp. 3.	8	Brewer's C Normal	0	0	4·93	4·88	—
			2½	47	1·85	3·32	2·54
			5½	79	0·40	1·64	2·32
	9	Same (acid treatment)	0	0	4·95	4·90	—
			3	26	3·24	4·10	2·38
			5½	45	2·06	3·34	2·29
	10	Same (alkali treatment)	0	0	4·93	4·88	—
			2½	33	2·70	3·84	2·51
			5½	56	1·42	2·88	2·36
Exp. 6.	11	Brewer's D 9°	0	0	4·23	4·17	—
			7	25	2·61	3·67	3·80
			22	70	0·61	1·91	2·48
	12	20°	0	0	4·79	4·72	—
			4	37	2·40	3·57	2·48
	13	31°	0	0	4·94	4·86	—
			4	48	1·90	3·23	2·33
			6	70	0·77	2·17	2·30
	14	8°	0	0	4·13	4·07	—
			23	31	2·34	3·29	2·67
			52	71	0·536	1·85	2·59
	15	34°	0	0	4·13	4·07	—
			4	38	2·04	3·02	2·36
			7½	71	0·64	1·76	2·22
	16	Sauterne (isolated 26. x. 29) 8°	0	0	4·09	3·98	—
			32	43	3·90	0·72	0·028
	17	34°	0	0	4·09	3·98	—
			5	50	3·73	0·27	0·034
	18	Sauterne 7°	0	0	9·26	2·06	—
			8	13	8·82	1·07	0·074
			19	25	8·26	0·22	0·051
	19	20°	0	0	9·99	2·21	—
			8	22	8·99	0·50	0·071
	20	31°	0	0	10·35	2·28	—
			8	12	9·79	1·29	0·098

1. 100 cc. of 0·146 *M* sucrose + 2·0 g. of pressed yeast + 4 cc. chloroform.
2. 100 cc. of 0·146 *M* sucrose + 100 cc. of 0·146 *M* glucose + 4·0 g. of pressed yeast + 8 cc. chloroform.
3. 100 cc. of 0·146 *M* sucrose + 100 cc. of 0·146 *M* fructose + 4·0 g. of pressed yeast + 8 cc. chloroform.

25 cc. samples were withdrawn and the reaction stopped by addition to 2 cc. of *N* NaOH and 1 cc. of alumina cream.

Table II. *Inhibition of inversion of sucrose by yeasts in the presence of glucose and fructose.*

			Polarimetric readings (2 dm. tube) at 20°			
			Sucrose + glucose		Sucrose + fructose	
			(0·146 *M*)	(0·146 *M*)	(0·146 *M*)	(0·146 *M*)
	Time (min.)	Sucrose (0·146 *M*)		Inhibition %		Inhibition %
Brewer's yeast	0	+6·09°	+5·57°	—	−1·41°	—
	60	+4·09°	+4·08°	25·5	−2·23°	59
	104	+3·10°	+3·37°	26·3	−2·73°	56
	149	+2·10°	+2·68°	27·5	−3·35°	51
Sauterne yeast	0	+5·88°	+5·38°	—	−1·36°	—
	34	+3·62°	+3·75°	28	−1·99°	72
	60	+2·02°	+2·63°	29	−2·92°	60

Exp. 3. *Selective fermentation by yeast weak in saccharase.*

Washed pressed brewer's yeast was treated with 0·2 *N* sulphuric acid for one hour and its saccharase value substantially reduced. Another portion suffered similarly by treatment with 0·05 *N* sodium hydroxide. A third portion was steeped in water. Selective fermentations were performed with the three yeasts immediately after washing. The results appear in Table I, Exps. 8, 9, 10.

Exp. 4. *Influence of saccharase on selective fermentation by zymin, prepared from brewer's yeast.*

To 200 cc. of a solution of the mixed sugars were added 10 g. of zymin; after mixing the solution was divided into two equal portions and a saccharase preparation added to one, and the same volume of boiled preparation to the other. A drop of toluene was added to each, and fermentation allowed to proceed at 25°. After a suitable gas evolution had occurred, fermentation was arrested by clearing with alumina cream, boiling and clearing afresh. The residual solution was analysed as for the yeast fermentations, and finally fermented to completion with living yeasts and again analysed, the latter results being deducted from the previous ones. In Exp. A, zymin from lager brewery yeast was used, and the saccharase preparation was a crude autolysate of yeast, too old to contain any co-zymase. In Exp. B, top brewery yeast zymin and a purified saccharase obtained from yeast autolysate by adsorption with aluminium hydroxide (Willstätter and Kraut) and eluted with dilute ammonia.

Table III. *Selective fermentation by zymin with and without added saccharase.*

	Saccharase preparation		Sugars g. per 100 cc. Glucose	Fructose
A. Lager yeast zymin	Crude yeast autolysate	Initial conc.	3·80	3·80
		Fermented by zymin	0·70	0·35
	Control	Initial conc.	3·80	3·80
		Fermented by zymin	0·62	0·28
B. Top yeast zymin	Purified saccharase	Initial conc.	3·33	3·02
		Fermented by zymin	1·97	0·18
	Control	Initial conc.	3·33	3·02
		Fermented by zymin	2·55	0·30

Exp. 5. *Velocity of fermentation of glucose and fructose in separate solutions.*

This was measured at 30° in the apparatus devised by Slator [1906]. The highest rate observed in the experiments with both brewer's and Sauterne yeasts was with fructose at 3·5 % in the fermenting mixture, and this was adopted as a standard in each case. The results, expressed in terms of these standards as 100 are given in Table IV. Autolysis rates have been deducted.

Table IV. *Relative rates of fermentation of glucose and fructose at 30°.*

	Sugar g. per 100 cc. of fermenting liquid	Glucose	Fructose
Brewer's yeast	0·20	51	31
	0·40	76	52
	1·00	89	90
	3·50	95	100
Sauterne yeast	0·20	—	43
	0·40	10	75
	0·50	15	79
	1·00	34	88
	3·50	54	100
	9·00	65	95
	10·50	71	87

Exp. 6. *Selective fermentations of glucose and fructose at different temperatures.*

In this experiment it is desirable to avoid either excessively fast or slow fermentations. In the former case the fermentation may be fast enough to compare with the rate of mutarotation of glucose, and in the latter, prolonged exposure to different temperatures may cause variations in the character of the yeast. Samples were withdrawn from the fermentations at intervals by rapid centrifugation followed by filtration. The procedure was as in Exp. 1, except that the reducing sugars were estimated by the Hagedorn-Jensen method as modified by Hanes, using the table of values furnished by Sobotka and Reiner [1930, 1]. In the case of fermentations with Sauterne yeast it was found desirable to start with a much higher proportion of glucose than of fructose, otherwise experimental errors are unduly large, and the appropriate juncture at which to withdraw a sample is difficult to judge. The results are

given in Table I, Exps. 11 to 20. The concentrations used in Nos. 18–20 were chosen with due regard to the results of Exp. 5.

Exp. 7. *Mutarotation of glucose solution when partly fermented by yeast.*

The experiments with glucose were performed by much the same procedure as was adopted by Willstätter and Sobotka [1922] except that the percentage of sugar fermented was determined by a separate withdrawal, and the addition of chloroform to arrest fermentation was omitted. About 200 g. of well-washed pressed yeast were added to an equal weight of 15 % glucose solution, and fermentation was allowed to proceed at 30° with frequent stirring. After a suitable time a few cc. of *N* acetic acid and a quantity of powdered ice were added and the liquid filtered rapidly through a large filter previously layered with alumina cream (free from salts, and neutral). The first runnings were rejected. The rotation could be read within two or three minutes of zero time, the latter being taken as the mean of the time of commencement and completion of filtration of the solution required to fill the observation tube. Fermentation was still proceeding in the funnel and was only arrested as the liquid passed through the filter. The acetic acid was added to ensure the attainment of a p_H of about 3·6 at which the rate of mutarotation is at a minimum. The fermentations with Sauterne yeast were performed at 20° and no ice added. It was found that less error was involved in this way, since when glucose is suddenly chilled a slow fall in rotation follows. The values of the constant $K_{\alpha/\beta}$ were calculated as by Willstätter and Sobotka, assuming $[\alpha]_D$ for α-glucose + 110°, for β-glucose + 19° and for the equilibrium solution + 52·6°.

Table V. *Mutarotation of glucose solutions partly fermented by yeast.*

	Polarimeter readings							
% sugar fermented	Initial (extrapolated from $t_1, t_2, \ldots$)	t_1	t_2	t_3	t_4	Final t_∞	$[\alpha]_D$ at time of arresting fermentation	$K_{\alpha/\beta}$
Brewer's yeast:		6 min.	12 min.	60 min.				
28·7	9·67°	9·73°	9·77°	9·88°	—	10·20°	+49·8°	1·53
		3 min.	5 min.	60 min.				
31·9	9·12°	9·24°	9·28°	9·46°	—	9·74°	+49·2°	1·47
		3 min.	14 min.	27 min.	100 min.			
38·6	8·20°	8·28°	8·38°	8·44°	8·58°	8·78°	+49·1°	1·38
		3 min.	14 min.		107 min.			
41·3	7·80°	7·90°	8·05°	—	8·19°	8·40°	+48·8°	1·38
Sauterne yeast:		4 min.	45 min.	100 min.	145 min.			
—	12·31°	12·29°	12·19°	12·14°	12·09°	11·99°	+54·0°	—
		3 min.	45 min.	100 min.				
—	11·60°	11·58°	11·52°	11·50°	—	11·25°	+54·2°	—
		4 min.	48 min.					
—	5·75°	5·73°	5·68°	—	—	5·56°	+54·4°	—

Exp. 8. *Mutarotation of fructose solution when partly fermented by yeasts.*

The procedure was as for glucose, with the following differences. In working with fructose much greater speed of observation is necessary than with glucose owing to the greater rate of mutarotation. It is also necessary to keep the temperature absolutely constant throughout fermentation, filtration and polarimetric observations. The experiments were performed at laboratory temperature, about 10°. It was not possible to calculate the values of K since the proportions of the constituents of fructose solution at equilibrium are unknown. Should the latter information become available, the data here presented may be of possible use in this connection.

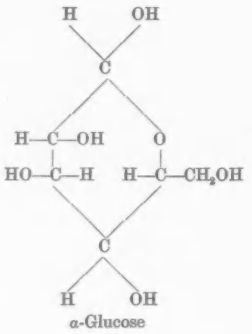
Table VI. *Mutarotation of fructose solutions partly fermented by yeast.*

		Polarimeter readings					
% sugar fermented	Initial (extrapolated from $t_1, t_2, \ldots$)	t_1	t_2	t_3	t_4	Final t_∞	$[\alpha]_D^{20°}$ at time of arresting fermentation
Brewer's yeast:		2 min.	4 min.	17 min.	38 min.		
13	9·37°	9·30°	9·27°	9·10°	8·98°	8·98°	−97·1°
		2 min.	5 min.	21 min.	40 min.		
21	8·55°	8·46°	8·38°	8·20°	8·14°	8·14°	−97·4°
		2 min.	5 min.	15 min.	36 min.		
25	8·12°	8·05°	7·99°	7·86°	7·79°	7·78°	−97·1°
		2 min.	4 min.	23 min.	50 min.		
54	4·90°	4·81°	4·76°	4·57°	4·52°	4·51°	−100·8°
		2 min.	5 min.	20 min.	90 min.		
56	5·06°	4·94°	4·84°	4·66°	4·64°	4·62°	−101·8°
Sauterne yeast:		4 min.	8 min.	24 min.			
44	6·00°	6·05°	6·08°	6·12°	—	6·14°	−90·8°
		3 min.		42 min.			
50	13·20°	13·26°	—	13·54°	—	13·60°	−90·3°
		2 min.	5 min.	25 min.			
50	8·56°	8·64°	8·73°	8·93°	—	8·96°	−89·0°
		2 min.	3 min.	28 min.	75 min.		
57	7·10°	7·16°	7·19°	7·62°	7·65°	7·72°	−85·6°

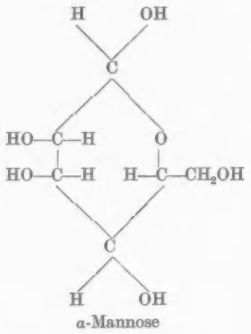
NOTE ON THE CONFIGURATIONS OF GLUCOSE, MANNOSE AND FRUCTOSE.

In view of the foregoing hypothesis, it is interesting to compare the configurational formulae of the three hexoses for which yeasts of the brewer's yeast type are supposed to be specific. It will be seen that, apart from the reducing carbon atom and its attachments, the three hexoses, glucose, mannose and γ-fructose are identical in configuration. The suggestion put forward by E. F. Armstrong that the three fermentable hexoses owe this property to certain features of their configurations which they have in common, could be modified to fit the hypothesis now put forward. Normal fructose has little

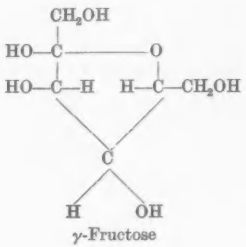
in common with the other three hexoses. The formulae of Haworth have been adopted.



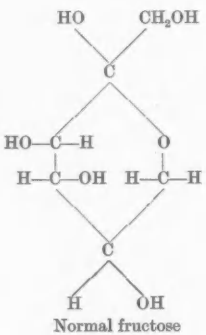
α-Glucose



α-Mannose



γ-Fructose



Normal fructose

Summary.

1. The factor $K_{G/F}$ for brewer's yeast was not appreciably influenced by the relative proportions of glucose and fructose in the original solution.

2. The factor was not influenced by modification of the saccharase activity of brewer's yeast by the method of Willstätter, Lowry and Schneider.

3. The saccharase of Sauterne yeast resembled that of brewer's yeast in that it was inhibited more strongly by fructose than by glucose.

4. The addition of saccharase to a mixture of glucose and fructose undergoing fermentation by zymin (brewer's) did not affect the selectivity.

5. The factor $K_{G/F}$ for brewer's yeast decreased with rise in temperature of fermentation, whilst that for Sauterne yeast increased.

6. Brewer's yeast ferments glucose faster than fructose in separate solutions when the concentration of sugar is less than 1 %. Sauterne yeast ferments fructose faster than glucose in separate solutions at all concentrations up to 10 %, but especially at low concentrations.

7. The mutarotation of partly fermented solutions of the sugars is for

glucose by brewer's yeast in the positive direction, glucose by Sauterne yeast in the negative, fructose by brewer's yeast in the positive, and fructose by Sauterne yeast in the negative direction.

8. The hypothesis is advanced that brewer's yeast and most yeasts are specific for that form of fructose which is present in small proportion, but which increases with temperature, possibly a γ-form, whereas Sauterne yeasts are specific for the normal form.

In conclusion I wish to express my thanks to Dr W. O. Kermack for the interest he has shown in this work, and for useful criticisms of the theories.

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XXXII. THE PROTEOLYTIC ENZYMES OF GREEN MALT.

II. ACTION OF THE PROTEINASE ON EGG-ALBUMIN, CASEINOGEN, EDESTIN AND FIBRIN AT DIFFERENT REACTIONS.

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Definite progress in the direction of clarifying our ideas on the nature of the proteolytic enzymes of green malt has been made in the past two years, during which no less than six papers appeared [Mill and Linderstrøm-Lang, 1929; Lüers and Malsch, 1929; Hopkins, 1929; Linderstrøm-Lang and Sato, 1929, 1, 2; Hopkins and Burns, 1930]. In their main conclusions these authors agreed that green malt contains (1) a protease or proteinase (to adopt the nomenclature of Grassmann) which appears to attack edestin, gelatin, crystalline egg-albumin and Witte peptone, and (2) at least one peptidase which attacks the dipeptide leucylglycine. According to Linderstrøm-Lang and Sato [1929, 2] there is a second peptidase present, which attacks alanylglycine and, unlike the previous one, is not inhibited by phosphates. The present communication is concerned with the activities of the proteinase.

It seems desirable, in investigations of the relations between enzyme activity and p_H, to use as purified a preparation of the enzyme as possible. Certain difficulties however present themselves. The yield of proteinase in the preparations obtained by the adsorption and elution methods so far tried is rather low, and the quantity of mineral salts present in the elutions is sufficient to influence the properties of the substrates. For instance, it was shown by Michaelis and Szent-Györgyi [1920] that the ions of certain salts influence the p_H of maximum precipitation of caseinogen. This is in agreement with certain observations of Hardy in connection with globulins. The present authors have also observed that phosphates and citrates, the former of which are used for elution of the enzyme, and both of which are used as buffers to stabilise p_H, inhibit the activity of the proteinase at p_H 4·7, but whether this is due to a cause such as that observed by Michaelis and Szent-Györgyi or to some other inhibitive effect was not investigated. For the present, the activity of the proteinase has been investigated using ordinary cold aqueous extract of green malt as the active agent, and in the absence of added buffers except in Exp. 1. The main object of the work was to ascertain

if the proteinase attacks its protein substrates optimally at their isoelectric points, *i.e.* is specific for the zwitterions, as suggested by Willstätter, Grassmann and Ambros [1926], in the cases of papain and bromelin.

In allowing the enzyme to act at 40° or thereabouts, it is necessary to adopt a short time of action, since the enzyme, in the presence of malt extract alone, is susceptible to fairly rapid inactivation or destruction when maintained at this temperature at a reaction outside the range of p_H 3·8 to 6·0. This was investigated by Hopkins and Burns [1930], who preserved an aqueous extract of green malt with toluene at various reactions at 40°. It was found that the proteinase is destroyed in the presence of free alkali (hydroxyl ions) and of excessive acidity, its optimum stability being at p_H 4·4 to 5·0, or near the isoelectric point (presumably) of the malt extract proteins. The activity was reduced to one-half in 24 hours at p_H 6·0, and in 48 hours at p_H 3·8. In order to avoid any displacement of the p_H of the optimum activity, and any serious distortion of the p_H-activity curves, the experiments recorded here were performed by allowing the enzyme to act on the substrates over a range of p_H for 8 hours. Except in the case of edestin, the proteolysis was measured by the amount of protein rendered soluble or non-coagulable, as the case may be, at its isoelectric point. This eliminated the measurement of any action due to the peptidases. The results may be briefly stated here.

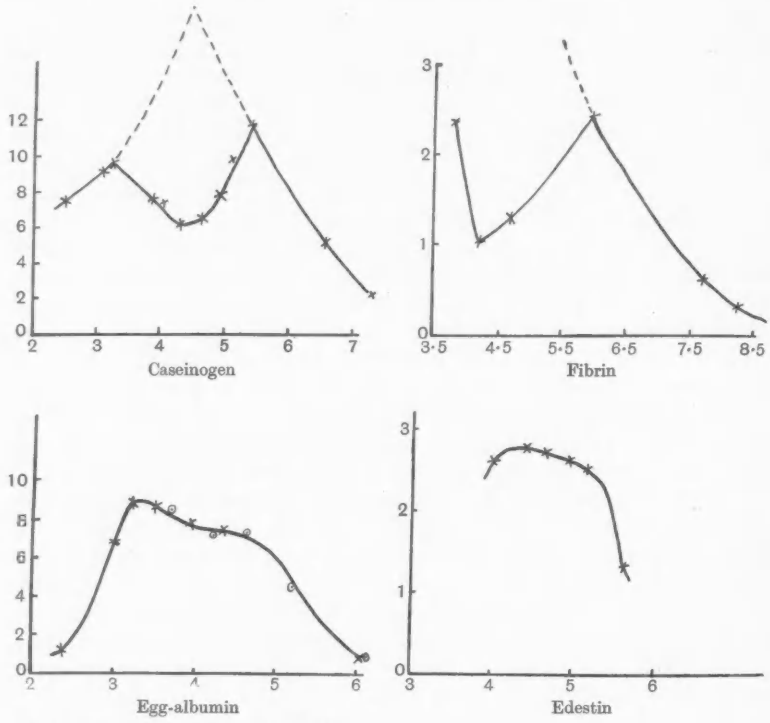
Egg-albumin. It was found that, at p_H 4·6 and 46°, proteolysis was proportional to time up to 6 hours or more (Exp. 1). The optimum activity was exhibited at p_H 3·3 in the presence of phosphate-citrate buffers (Exp. 2) and at a point more acid than p_H 3·8 in the absence of added buffers (Exp. 3). The isoelectric point of egg-albumin is at p_H 4·8 according to Sørensen. The activity at p_H 3·87 in the absence of added buffers was 1·23 times that at p_H 4·8 (Fig. 1).

Caseinogen. At 46° and p_H 5·1 proteolysis was proportional to time up to 9 hours (Exp. 5). Two optima of activity were observed at p_H 3·35 and 5·57, *i.e.* on each side of the isoelectric point, which is 4·62 according to Michaelis and Pechstein [1912] (Exps. 6 and 7).

Edestin. The relation between proteolysis and p_H for edestin was investigated by Mill and Linderstrøm-Lang [1929] who found the optimum to be at p_H 4·3. This was obtained in the presence of chlorine ions, the protein being in solution in hydrochloric acid or sodium chloride. These results have been confirmed using a shorter period of time for the reaction (Exp. 9). In view of the work of Michaelis and Szent-Györgyi [1920] already referred to, the experiment was repeated using acetic acid instead of hydrochloric acid (Exp. 10). The point of maximum precipitation of edestin is unaffected by sodium acetate, whereas in the presence of sodium chloride it is shifted in the acid direction. It was found, however, that proteolysis increased with acidity up to the maximum attainable with acetic acid as the sole acid present.

Fibrin. At 46° and p_H 4·8 proteolysis was proportional to time for 2 hours only (Exp. 12). Two optima of activity were observed at p_H 3·8 and 6·0, *i.e.*

on each side of the zone of maximum precipitation of the protein under the conditions obtaining. The isoelectric point of fibrin is generally stated to be about 6·8, for which reason fibrin was used. It was very difficult to dissolve, and, after solution had been effected under somewhat drastic conditions, the zone of maximum precipitation was at about p_H 4·8.



Ordinates. Proteolysis as cc. $N/10$ HCl.
Abscissae. p_H.
The broken lines represent the forms that the curves would presumably take, if the substrates did not flocculate within the zones of minimum solubility.
Readings from Table II are represented by crosses on the egg-albumin curve, readings from Table III (after reduction to appropriate values of the ordinate) are represented by encircled dots.

The diagram illustrates the results, ordinates representing proteolysis, the abscissae p_H.

Discussion of the results.

The p_H optima of proteinase activity under the conditions adopted do not coincide with the isoelectric points of the respective substrates. In the cases of caseinogen and fibrin, the protein being almost insoluble over a zone of p_H including the isoelectric point, it is only to be expected that proteolysis

would be less vigorous here, since the substrate is partly or wholly in suspension, and the concentration in solution is low enough to limit the rate of enzyme action. This cannot be avoided, since, if the very small fraction of protein which remains in solution at the point of maximum precipitation were alone used as substrate, and proteolysis measured by the Willstätter or formaldehyde titration (which would be necessary in this case), we should probably be using neither caseinogen nor fibrin as substrate, but a small fraction of abnormal and possibly contaminated material. The p_H activity curves of both caseinogen and fibrin strongly suggest that, but for the insolubility of the substrate within this zone, the proteolytic action would have increased to an optimum within the zone, and in the case of caseinogen this would have approximated to the isoelectric point. However, egg-albumin, which is soluble at all reactions investigated, gives results indicating that the optimum is not at the isoelectric point (4·8) but at p_H 3·3 to 3·6. Edestin, although held in solution fairly completely by sodium chloride or acetate at all reactions investigated, showed greater susceptibility to proteolysis as acidity increased from p_H 5·5, whereas the isoelectric point of edestin is at p_H 5·5 to 6·0.

These varying results for the four substrates suggest much speculation on such lines as the state of molecular aggregation and osmotic pressure of the respective proteins at their isoelectric points and elsewhere, and the presence or otherwise of zwitterions or of un-ionised molecules in the various cases.

However, one observation is sufficiently striking to merit notice. It will be seen from the electrometric titration data given in Tables IV, VIII, XI and XIV that the p_H optimum for proteolysis of each protein approximates to that of maximum buffering power. The latter is given in the Tables, expressed as the Van Slyke coefficient $\Delta A/\Delta\ p_H$ or $\Delta B/\Delta\ p_H$, where ΔA and ΔB are increments of added acid and base, and $\Delta\ p_H$ the corresponding change in p_H. The p_H range, corresponding to the maximum values of this coefficient for each protein, together with the results derived from the electrometric titrations of other observers, are summarised in Table XV. The only appreciable discrepancy is in the case of edestin, but even here the p_H optimum of proteolysis found is much nearer to that of buffering than to the isoelectric point. It seems that, when the p_H of maximum buffering power of the protein substrate falls within the range of activity of the malt proteinase, the optimum proteolysis will occur at or near that p_H. In the case of the proteins possessing an insoluble zone, the optimum is necessarily outside this insoluble zone. The proteinase appears to act on ionised protein, on both sides of the isoelectric point, and it is quite possibly specific for zwitterions where these exist. Further speculation is at present premature.

Experimental.

Preparation of the malt extract. Air-dried green malt was finely ground, extracted with 3 times its weight of water for 12 hours at laboratory tem-

perature and filtered. The extract used in the experiments, in which the formaldehyde titration was employed to measure proteolysis, was prepared from kiln-dried malt, which is unlikely to contain any quantity of the peptidases.

The substrates were crystalline egg-albumin (Merck), edestin from hempseed (Merck), caseinogen (a commercial product purified by Hammarsten's process), and fibrin prepared directly from blood and preserved in alcohol.

The buffer solution used in Exp. 2 was McIlvaine's phosphate-citrate, as described by Clark [1928].

All hydrogen ion measurements were performed by means of the quinhydrone electrode.

Exp. 1. *Egg-albumin. Kinetics.* 100 cc. of 6 % albumin + 20 cc. of acetate buffers (sodium acetate and acetic acid, $N/2$ with respect to each), p_H 4·6 + 50 cc. of malt extract, mixed and incubated at 46°. Control, the same with boiled malt extract. 25 cc. withdrawn at intervals and added slowly to 60 cc. of boiling water and boiled for 15 minutes, filtered, washed with boiling water, and total nitrogen determined in filtrate by the Kjeldahl process.

Table I.

Time (hours)	3	6	12	24	36
Action, cc. $N/10$ HCl (control deducted)	3·5	7·2	9·8	10·5	10·9

Exp. 2. *Egg-albumin. Proteolysis at different reactions.* 15 cc. of albumin + 15 cc. of malt extract + 10 cc. of phosphate-citrate buffer solution of appropriate p_H + $N/10$ HCl or NaOH as required + water to a total volume of 60 cc. After 8 hours at 46°, 30 cc. were withdrawn and coagulated as in Exp. 1, after addition of the required volume of $N/10$ NaOH or HCl. The latter was firstly calculated, and then a *pro rata* portion added to 20 cc. of the remaining solution and the resulting p_H tested to confirm that it was 4·8. The remaining portion of the reaction mixture was used for the determination of its p_H.

Table II.

p_H of reaction mixture	2·25	2·97	3·34	3·69	3·74	4·11	4·40	6·13	6·69	7·14	7·52	7·77
p_H of control	2·25	2·97	3·30	3·69	—	—	—	6·13	6·70	7·14	7·50	7·73
Proteolysis, cc. $N/10$ HCl (controls deducted)	1·0	5·5	8·6	8·0	7·9	7·0	6·4	1·2	0·8	0·6	0·5	0·3

Exp. 3. *Egg-albumin. Proteolysis in absence of added buffers.* This experiment was performed exactly as the previous one except that the phosphate-citrate buffers were omitted. All withdrawals were treated so as to contain the same concentration of NaCl at the time of coagulation.

Table III.

p_H of reaction mixture	3·87	4·32	4·73	5·28	6·18
p_H of control	3·73	4·30	4·73	5·28	6·19
Proteolysis, cc. $N/10$ HCl (controls deducted)	8·5	7·0	6·9	5·0	1·5

Exp. 4. *Electrometric titration of the egg-albumin.* Table IV gives the volumes of $N/10$ HCl or NaOH added to 1 g. of protein, and the p_H resulting. The buffer coefficients were calculated according to the formula of Van Slyke $\Delta A/\Delta p_H$ or $\Delta B/\Delta p_H$.

Table IV.

$N/10$ acid cc.	0·0	0·5	1·0	1·5	2·0	2·5	3·0	3·5	4·0	5·0
p_H	4·89	4·55	4·30	4·06	3·87	3·67	3·49	3·31	3·09	2·64
$\Delta A/\Delta p_H$		1·47	2·00	2·08	2·63	2·50	2·78	2·78	2·27	2·22
$N/10$ base cc.	0·0	0·5	1·0	1·1	1·2	1·3	1·4			
p_H	4·89	5·31	6·03	6·27	6·55	6·83	7·09			
$\Delta B/\Delta p_H$		1·19	0·70	0·41	0·35	0·35	0·38			

Exp. 5. *Caseinogen. Kinetics.* 2 g. of caseinogen, suspended in 100 cc. of $N/20$ NaOH until dissolved, were brought to p_H 5·1 with $N/2$ HCl. 50 cc. of malt extract previously adjusted to p_H 5·1 were added, and the mixture incubated at 46°. At intervals 20 cc. were withdrawn, added to 20 cc. of acetate buffer solution (p_H 4·6), filtered, and total nitrogen determined on the filtrate. The control was treated similarly using boiled malt extract.

Table V.

Time (hours)	3·0	6·5	9·0
Action, cc. $N/10$ (control deducted)	4·2	7·8	10·7

Exp. 6. *Caseinogen. Proteolysis at different reactions.* 20 cc. of caseinogen (2 % in $N/20$ NaOH) + 15 cc. of malt extract + 19 cc. of $N/10$ HCl + respectively 0·0, 3·0, 5·0, 7·0, 8·0, 11·0 cc. of $N/10$ NaOH + water to 80 cc. Of this 20 cc. were withdrawn for p_H determination, and the remainder incubated at 46° for 8 hours. To each was now added the requisite volume of $N/10$ NaOH or HCl to bring to p_H 4·6 + water to the same total volume. Total nitrogen was determined on 50 cc. of the filtrate.

Table VI.

p_H of reaction mixture	2·58	2·97	3·35	3·82	4·10	5·34
Proteolysis, cc. $N/10$ HCl (controls deducted)	7·42	8·75	9·60	7·70	7·50	9·66

Exp. 7. *Caseinogen. Proteolysis in presence of acetate.* The procedure was exactly as in the previous experiment except that acetic acid was used instead of HCl.

Table VII.

p_H of reaction mixture	3·91	4·41	4·70	5·00	5·56	6·50	7·40
Proteolysis, cc. $N/10$ HCl (controls deducted)	7·6	6·2	6·6	7·9	11·7	5·16	2·2

Exp. 8. *Caseinogen. Electrometric titration.* Table VIII gives the volumes of $N/10$ HCl added to 0·2 g. of caseinogen in $N/10$ NaOH, and the resulting p_H. The buffer coefficients are also given.

Table VIII.

$N/10$ HCl cc.	8·0	8·4	8·6	8·8	9·0	9·2	9·4	9·6	9·8
p_H	8·37	6·89	6·47	6·31	5·93	5·49	5·15	4·71	4·16
$\Delta A/\Delta p_H$		0·27	0·48	1·25	0·53	0·45	0·58	0·45	0·36
$N/10$ HCl cc.		10·0	10·5	11·0	11·5				
p_H		3·80	3·36	3·00	2·60				
$\Delta A/\Delta p_H$		0·55	1·14	1·40	1·25				

Exp. 9. *Edestin. Proteolysis at different reactions.* 1 g. of edestin was suspended in 50 cc. of $N/10$ HCl until dissolved. 10 cc. of the solution + 15 cc. of malt extract + varying volumes of $N/10$ NaOH and water to total volume 40 cc. The mixtures were incubated at 37° for 14 hours. After withdrawing 10 cc. for the determination of p_H, the remaining 30 cc. were subjected to the formaldehyde titration. The controls were performed on malt extract + water, at the same values of p_H, and a control for edestin alone.

Table IX.

p_H of reaction mixture	4·0	4·4	4·6	4·9	5·1	5·5
Proteolysis, cc. $N/10$ NaOH (controls deducted)	2·65	2·80	2·75	2·70	2·55	1·50

Exp. 10. *Edestin. Proteolysis in presence of acetate.* The process was exactly as in Exp. 9 except that acetic acid was used instead of HCl. There was comparatively little protein in suspension in the reaction mixture, but a little was unavoidable in some cases.

Table X.

p_H of reaction mixture	4·25	4·40	4·64	4·82	5·05
Proteolysis, cc. $N/10$ NaOH (controls deducted)	1·65	1·40	1·35	1·25	1·20

Exp. 11. *Edestin. Electrometric titration.* Table XI gives the volumes of $N/10$ NaOH added to 0·05 g. of edestin, and the p_H resulting. The protein was dissolved in $N/10$ HCl.

Table XI.

$N/10$ NaOH cc.	4·0	4·2	4·4	4·5	4·6	4·7	4·8	4·9
p_H	2·41	3·37	3·54	4·04	4·47	4·82	6·58	8·58
$\Delta A/\Delta p_H$		0·21	1·18	0·20	0·23	0·28	0·06	0·05

Exp. 12. *Fibrin. Kinetics.* 2 g. of fibrin were dissolved in $N/5$ NaOH with heat, and brought back to p_H 6·8 and 4·6 in two portions. There was no precipitation at p_H 6·8, but precipitation took place at p_H 4·6. $N/2$ acetic acid was used for the purpose. Two experiments were performed, one on each solution, using 50 cc. of fibrin + 30 cc. of malt extract + water to 130 cc. Controls were treated similarly, using boiled malt extract. The reaction was checked by withdrawing 20 cc. at intervals, adding the required volume of $N/2$ acetic acid to bring to maximum precipitation and filtering. Total nitrogen was determined on the filtrates.

Table XII.

Time (hours)	1	2	4	
Action, cc. $N/10$ (controls deducted).	0·5	1·2	1·5	At p_H 4·5
	—	0·6	0·8	At p_H 6·9

Exp. 13. *Fibrin. Proteolysis at different reactions.* 5 g. of fibrin + 50 cc. of $N/2$ acetic acid were heated to just below boiling, allowed to stand for a time in the cold, filtered and the swollen fibrin dissolved in 300 cc. of $N/8$ NaOH by heating. By this means the fibrin was dispersed with less drastic treatment than in the previous experiment. It still precipitated, however, at p_H 4·8 on neutralisation, instead of p_H 6·8 which is the isoelectric point of fibrin. 20 cc. of fibrin + 15 cc. of malt extract + the required volumes of acetic acid or sodium hydroxide + water to total volume 80 cc. After 5 hours at 46°, 40 cc. were withdrawn and brought to p_H 4·8 by addition of the necessary volumes of acetic acid or NaOH, etc. as in Exp. 12.

Table XIII.

p_H of reaction mixture	3·81	4·20	4·58	5·00	6·03	7·59	8·27	9·00
Proteolysis, cc. $N/10$ HCl (controls deducted)	2·3	0·9	1·1	1·6	2·3	0·6	0·3	0·0

Exp. 14. *Fibrin. Electrometric titration.* The fibrin was dispersed as in Exp. 12. Table XIV gives the volume of $N/10$ HCl added to 0·1 g. of fibrin in $N/5$ NaOH. The buffer coefficients are also given.

Table XIV.

$N/10$ HCl cc.	9·0	9·3	9·5	9·6	9·8	9·9	10·0	10·2	10·4
p_H	7·60	6·22	5·91	5·46	4·86	4·16	3·80	3·11	2·89
$\Delta A/\Delta p_H$	0·22	0·64	0·22	0·33	0·14	0·28	0·29	0·91	

Table XV. *Summary of* p_H *values corresponding to maximum values of buffer coefficients and proteinase activity.*

Protein	p_H range of maximum: Buffer coefficient	Activity of proteinase
Egg-albumin	3·31–3·67 (present communication) 3·40–3·53 [Hendrix and Wilson, 1928] 3·10–3·50 [Loeb, 1921]	3·3–3·6
Caseinogen	3·00–3·35 and 6·31–6·47 (present communication) 3·10–3·41 and 6·10–6·52 [Loeb, 1921]	3·0–3·35 and 5·57
Edestin	3·37–3·54 (present communication) 3·30–3·60 [Hitchcock, 1922]	4·25–4·4
Fibrin	3·11–4·16 (present communication) and 5·91–6·22	3·81 or less and 6·0

SUMMARY.

1. The proteinase of green malt acts at 46° optimally on crystalline egg-albumin at p_H 3·3 to 3·6, on caseinogen at p_H 3·35 and at 5·57, on fibrin at p_H 3·8 (or less) and at 6·0, and on edestin at 37° optimally at p_H 4·3.

2. These values of p_H approximate in most cases very closely to those of the maximum buffering power of the protein measured at 18°.

3. The proteinase appears to act on ionised protein on both sides of the isoelectric point, but there is no clear evidence that it is specific for zwitterions, although this is quite possible in the cases of those proteins in which zwitterions exist.

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XXXIII. CAROTENE AND VITAMIN A.
THE CONVERSION OF CAROTENE INTO VITAMIN A BY FOWL.

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It has now been established with a fair degree of certainty that carotene possesses vitamin A activity [Euler, Euler and Hellström, 1928; Moore, 1929, 1, 2, 1930; Collison, Hume, Smedley-MacLean and Smith, 1929; Kawakami and Kimm, 1929; Hume and Smedley-MacLean, 1930; Karrer, Euler and Rydbom, 1930]. It has also been shown that, in the rat, carotene is converted into the "classical" vitamin A of liver oils, characterised by the blue colour given with the antimony trichloride reagent (absorption band 610–630$\mu\mu$) and by the presence of an absorption band in the region of 325$\mu\mu$ [Capper, 1930; Moore, 1930]. Before any generalisations could be made it seemed desirable that similar experiments should be carried out with animals differing widely from the rat. For this reason, as well as for the fact that in the fowl the problem presents points of peculiar interest, the work to be described was undertaken.

It will be shown that the fowl as well as the rat can convert carotene into vitamin A; that the vitamin A requirements of the fowl are higher than those of the rat weight for weight, and that the liver oil is normally very much richer in vitamin A than is cod-liver oil.

Experimental.

Exp. 1. The pullets used were White Wyandottes which had originally been intended for another experiment, and a deficiency of vitamin A in the diet was in the nature of an unexpected accident. The basal diet consisted of:

Cereals	
Bran 2 parts, pollards 1 part, yellow maize meal 1 part, Sussex ground oats 1 part by weight...	78 %
Soya bean meal	18 %

Mineral mixture			
Steamed bone flour	10 parts by wt.		
Commercial potassium chloride ("muriate of potash")	2	„	
Common salt	1·5	„	
Sulphur	0·25	„	
Iron oxide	0·1	„	
Potassium iodide	0·01	„	4 %

A scratch grain consisting of whole wheat was fed at the rate of approximately 50 % of the total food consumed and oyster shell was supplied *ad lib.* as a source of lime.

Group A had been kept indoors in a hut glazed with vita glass and had received the basal diet only.

Group B had received the basal diet but in addition had had free access to an open air grass run.

Group C had been kept indoors in a hut glazed with ordinary glass and in addition to the basal diet had received natural cod-liver oil at the rate of about 2 % of their diet.

The birds had been put on these diets as day-old chicks, except that for the first 9 weeks separated milk replaced the mineral mixture. At the age of approximately 16 weeks while the birds of Groups B and C remained healthy and normal the majority of those of Group A began to decline in weight and were obviously unwell. Their sense of balance appeared to be disturbed and they walked with difficulty and with a staggering gait. Rachitic trouble was at first suspected but normal calcium and phosphorus figures were obtained by Mr R. H. Common for samples of their blood-serum, and when two of the birds were killed autopsy revealed normal ossification. On examination of their livers for the presence of vitamin A negative results were obtained both by the $SbCl_3$ test, and spectroscopically by the absence of any selective absorption in the region of 325 $\mu\mu$. The liver oils of birds of Groups B and C and of birds procured in the ordinary market all gave an intense blue colour with $SbCl_3$ and a well marked absorption band in the region of 325 $\mu\mu$.

In testing the livers for vitamin A the technique described by Moore [1930] was followed in the main. The liver was minced, mixed with about twice its bulk of 5 % KOH and left for several days at least. It was then extracted four times with ether, the ethereal extract washed three times with water, dried over anhydrous sodium sulphate and the ether evaporated off by slight warming under reduced pressure. The oil thus obtained was then dissolved in chloroform in such dilution that 0·2 cc. solution + 2 cc. $SbCl_3$ reagent gave between 3 and 10 blue units on a Lovibond tintometer. The "blue units" and "yellow units" were then calculated as described by Moore [1930].

The apparatus used for the determination of the absorption spectra

was that previously described [Capper, 1930]. The results are shown in Table I.

Table I.

No. of bird	Diet	Wt. of liver (g.)	Wt. of liver oil obtained (g.)	Total "blue units" found in liver	"Blue units" per g. of liver	Total "yellow units"
A 1	Basal only	11	0·15	0	0	—
A 2	,,	18	0·10	0	0	15
B 1	Basal and greenstuff	16	0·22	2,200	140	150
B 2	,,	24	0·19	48,100	2,000	6500
C 1	Basal and cod-liver oil	24	0·13	24,800	1,030	80
*C 2	,,	23	0·13	5,500	240	25
M 1	Birds procured in the open market	43	1·05	103,000	2,370	1060
M 2		33	0·90	41,200	1,250	375
M 3		27	0·50	375,000	10,100	—

* This value is probably too low as there was some loss due to emulsification in certain stages of the separation.

The large amount of vitamin A that may sometimes be found in the livers of fowls is shown by the values recorded for the liver of hen No. M 3 where the natural liver oil appears to be richer in vitamin A than the unsaponifiable residue of cod-liver oil, which generally gives about 300,000 blue units per g. of concentrate as compared with 750,000 blue units per g. of this liver oil. The high vitamin A content indicated by the $SbCl_3$ test was confirmed by the persistence of the absorption band in the region of 325$\mu\mu$ in the absorption spectrum and for M 1 by feeding tests on rats carried out at Cambridge by Dr Moore, who found the liver oil active as vitamin A at about the level indicated by these figures (see Fig. 5).

Nine birds of Group A were then selected for further experiment. All were confined in the same wooden house glazed with vita glass; three continued to receive the basal diet only, three received in addition cod-liver oil concentrate, while the remaining three received the basal diet + carotene dissolved in arachis oil. The cod-liver oil and carotene solutions were administered orally by means of a fountain pen filler. The growth curves and daily doses of these birds are shown in Fig. 1.

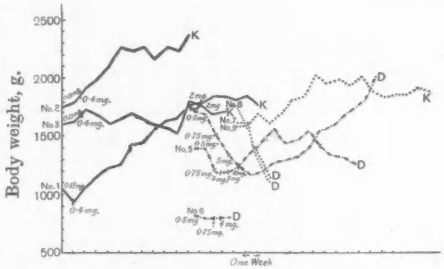

Fig. 1. ——— Birds receiving carotene.
—·—·— ,, cod-liver oil concentrate.
..... ,, neither carotene nor cod-liver oil.
The figures indicate the daily doses. D = died. K = killed.

The large vitamin A requirement of fowls was not realised at first and the birds continued to decline in weight and to become more helpless until the daily dose of cod-liver oil concentrate had been increased to about 10 mg. and of carotene to 0·5 mg. (The carotene used was prepared by Dr Moore from red palm oil, M.P. 168° (uncorr.).) Before the dose of carotene had been increased to 0·5 mg. No. 1 had so declined as to be unable to stand upright and lay helpless on its side; with the increased dose of carotene, however, recovery was most rapid, walking became possible within 24 hours and weight steadily increased. No. 4 had reached almost the same state before the dose of cod-liver oil concentrate had been increased to 10 mg. daily, after which recovery and growth were continuous and death after 16 weeks was due, not to any dietary deficiency, but to prolapsus of the oviduct. Nos. 5 and 1 eventually became broody and for this reason lost weight during the final weeks of the experiment. Of the control birds which continued to receive the basal diet only, Nos. 7 and 8 rapidly declined and died, No. 7 developing eye trouble resembling xerophthalmia. No. 9, which was the strongest bird at the beginning of the experiment, continued to live and at no time exhibited any pathological condition. Since the birds were confined together it may conceivably have received some vitamin A from the excreta, etc. of the birds receiving cod-liver oil concentrate or carotene or it is possible that it possessed lower requirements and utilised what it received more efficiently.

On examination the liver oils of all the birds that had received either cod-liver oil concentrate or carotene gave positive reactions for vitamin A with $SbCl_3$ and showed absorption bands at about 325$\mu\mu$, while the oils of the birds that had received the basal diet only invariably gave negative results both colorimetrically with $SbCl_3$ and spectroscopically. The results of the examination of the livers are shown in Table II.

Table II.

No. of bird	Daily dose during final weeks of exp.	Wt. of liver (g.)	Wt. of liver oil (g.)	Total "blue units" with $SbCl_3$ found in liver	"Blue units" per g. of liver	Total "yellow units" in $CHCl_3$
1	2 mg. carotene	32	0·24	8,350	265	20
2	1 mg. ,,	56	1·63	330	6	150
3	2 mg. ,,	23	0·28	3,700	160	150
4	10 mg. C.L.O. conc.	36	0·46	8,250	225	25
5	10 mg. ,,	20	0·18	10,500	525	25
6	4 mg. ,,	32	0·16	165	5	10
7	0	34	0·14	0	0	5
8	0	26	0·14	0	0	10
9	0	41	0·35	0	0	—

This experiment suggests strongly that the fowl as well as the rat can transmute carotene into vitamin A. At the same time it is in some respects open to criticism, and not completely satisfactory. Since the fowls used were approaching maturity, regular growth curves could not be expected; the diet was not sufficiently synthetic and birds receiving different diets were confined

in the same house. For these reasons a second experiment was carried out on more rigid lines.

Exp. 2. The basal diet used had the following composition:

Caseinogen (Glaxo physiological AB) ...	18 %
Agar	2
Yeast (dried)	15
Dextrin	60
Salt mixture	5

The salt mixture had the composition given by Hart, Halpin and Steenbock [1920].

Vitamin D was supplied by administering 2 drops (afterwards increased to 10 drops) of radiostol or an equivalent amount of ostelin daily. The food was supplied dry and drinking water was available *ad lib.* No litter was used but sand covered the floor of the houses.

Twelve White Wyandotte[1] chickens (Group A) were placed on this diet at the age of 6 weeks and confined in a wooden poultry house. Six more chickens (Group B) from the same batch were placed in a similar house and received the same basal diet with the addition of 0·5 mg. cod-liver oil concentrate daily.

For some weeks the Group B birds gained weight considerably faster than the others, as may be seen from the growth curves in Figs. 2 and 3, but after about 6 weeks both groups declined in weight and four of Group A and three of Group B died. (One bird in Group A died soon after being placed on the diet and has been omitted from consideration.) When examined for the presence of vitamin A the livers of all six gave negative results. It was then realised that the doses of cod-liver oil concentrate were insufficient and these were increased as shown in Fig. 2. It is interesting to note that only No. 7 exhibited eye trouble in any way resembling xerophthalmia, nor did any of them display the curious staggering walk shown by the birds in Exp. 1. The "third eyelids" of all the birds were partially closed and they had a generally dejected appearance. *Post mortem* examination of those that died revealed powdery white deposits which gave the murexide test for urates round the heart, liver and other organs, and the disease was diagnosed in the Animal Diseases Laboratories of the Northern Ireland Ministry of Agriculture as visceral gout. This condition would appear to be similar to, but more severe than, that found by Hart *et al.* [1924] in their work on the nutritional requirements of chicks.

The remaining birds of Group A were now given carotene dissolved in arachis oil. The daily doses and growth curves are shown in Fig. 3.

With Nos. 7, 10 and 13 the disease had progressed too far and they succumbed within 2 days. All the remaining birds in both groups now rapidly

[1] In a previous communication these chickens were by a slip reported as White Leghorns (Capper, *Nature*, 1930, **126**, 685). The opportunity is now taken to correct the error.

improved in appearance and at once began to increase in weight. Eventually Nos. 0, 2, 6 and 17 became fully mature birds, and when killed ossification was found to be good and, except for a certain paleness, the organs appeared

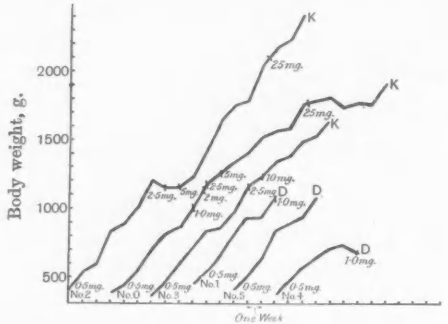

Fig. 2. Growth curves of birds receiving cod-liver oil concentrate. The figures indicate the daily doses. D = died. K = killed.

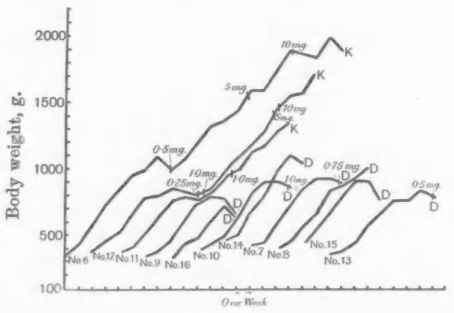

Fig. 3. Growth curves of birds receiving carotene and controls. The figures indicate the daily doses. D = died. K = killed.

normal and fully developed. No. 6 commenced to lay at the age of 23 weeks, having then been fed for 17 weeks on the synthetic diet and in the next month which followed before it was killed laid 20 eggs ranging in weight from 34·5 g. to 59 g. with an average weight of about 40 g. The shells of these eggs were brown in colour but the yolks were almost colourless, possessing only a faint yellow tinge when hard boiled.

The livers of all the birds were examined for the presence of vitamin A both colorimetrically by $SbCl_3$ and spectroscopically. In addition, representative liver oils were despatched to Cambridge in evacuated tubes and tested biologically for vitamin A on rats by Dr Moore. The results of the examination of the liver oils are shown in Table III.

Table III.

No. of chicken	Diet during final weeks	Wt. of liver (g.)	Wt. of liver oil (g.)	Total "blue units" found in liver	"Blue units" per g. of liver	Total "yellow units" in liver	Absorption spectrum
0	Basal + 25 mg. C.L.O. conc.	42	0·77	8,250	190	50	Band at 325 $\mu\mu$
1	Basal + 0·5 mg. C.L.O. conc.	30	0·22	0	0	8	No band near 325 $\mu\mu$
2	Basal + 25 mg. C.L.O. conc.	63	0·59	15,100	240	30	Band at 325 $\mu\mu$
3	Basal + 10 mg. C.L.O. conc.	34	0·23	11,000	320	15	—
4	Basal + 0·5 mg. C.L.O. conc.	22	0·16	0	0	8	No band near 325 $\mu\mu$
5	Basal + 0·5 mg. C.L.O. conc.	25	0·19	0	0	15	—
6	Basal + 10 mg. carotene	41	0·59	8,200	200	100	Band at 325 $\mu\mu$
7	Basal only	20	0·12	0	0	10	No band near 325 $\mu\mu$
8	,,	29	0·16	0	0	10	,,
10	,,	25	0·13	0	0	10	No band near 325 $\mu\mu$
11	Basal + 1 mg. carotene	28	0·24	250	9	29	—
13	Basal only	22	0·14	0	0	8	No band near 325 $\mu\mu$
14	,,	24	0·09	0	0	15	,,
15	,,	22	0·21	0	0	—	—
16	,,	17	0·08	0	0	10	No band near 325 $\mu\mu$
17	Basal + 10 mg. carotene	44	0·19	1,300	30	35	Band at 325 $\mu\mu$

The liver oils of chickens which had received the same diet gave similar absorption spectra. Typical examples of the absorption spectra of the liver oils are shown in Fig. 4, and in Fig. 5 are shown the growth curves and daily doses of the rats which were given the chicken-liver oils as a source of vitamin A.

It may be pointed out here that when the chickens were first placed on the synthetic diet, after having received a normal diet containing greenstuff, their beaks and shanks were highly pigmented, but after a few weeks on the carotenoid-free synthetic diet the yellow colour faded completely and even after receiving large doses of carotene daily for periods of up to 9 weeks no increase in pigmentation was noted, which is in agreement with the results obtained by Palmer and Kempster [1919] who found that xanthophyll was necessary for the pigmentation of the beak and shanks. In addition, while the liver oil of a hen on a normal diet was found to give deeply pigmented solutions in which absorption bands corresponding to those recorded for xanthophyll were observed, the liver oils of the birds which had received the synthetic diet + carotene gave solutions which were only slightly yellow in colour.

If we take 0·5 mg. carotene as the minimum daily requirement for a hen weighing 2000 g. and compare it with the minimum daily requirement of carotene (0·002 mg.) found by Moore [1930] for a rat weighing 100 g. it is

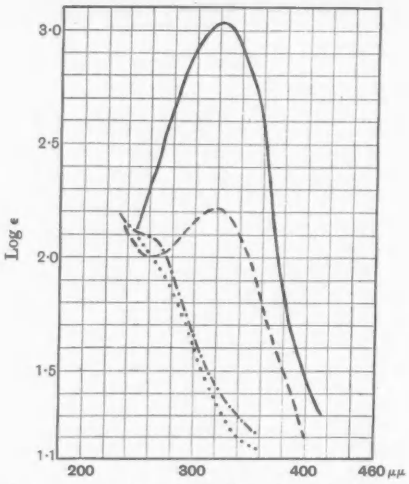

Fig. 4. ——— Liver oil of chicken No. 3 in chloroform.
— — — " " 6 "
—·—·— " " 8 "
····· " " 1 "

ϵ is defined by $\log I_0/I = \epsilon cd$, where c is the concentration of the liver oil in g. per cc., d is the cell thickness in centimetres.

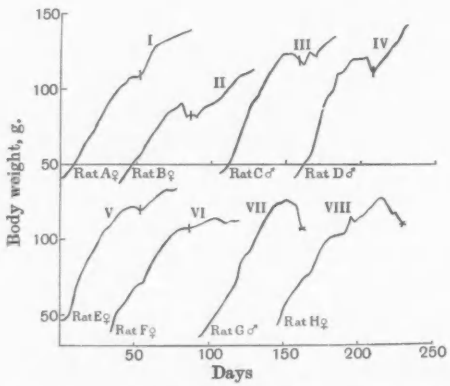

Fig. 5. Confirmation of the vitamin A activity of chicken-liver oils by rat-growth tests.
I and II. Liver oil of bird M 1 (Table I) 0·013 mg. daily = 1 B.U.
III. Liver oil of bird 3 (Table II) 0·2 mg. daily = 2 B.U.
IV. " " 0·4 " = 4 B.U.
V. " bird 6 (Table (III) 0·4 " = 5 B.U.
VI. " bird 11 (Table III) 2·0 " = 2 B.U.
VII and VIII. Negative controls.

Rats *A*, *B*, *E*, *G* received a basal diet containing Glaxo caseinogen 20 %, rice starch 60 %, palm kernel oil 15 %, salt mixture 5 %, supplemented by 10 % of dried yeast and one drop of radiostol daily. The basal diets of rats *C* and *D* differed in containing a similar amount of arachis oil in place of palm kernel oil and 7·5 % of marmite extract in place of dried yeast, the basal diets of rats *F* and *H* in containing additional starch in place of the fat component. The test doses of chicken-liver oils were diluted in arachis oil and administered at the points indicated in the figure by a vertical line.

seen that the vitamin requirements of the fowl appear to be greater than those of the rat weight for weight.

As misleading results have been obtained in the past by other workers through the use of a solvent in which the carotene altered, it may be stated that the solution of carotene in arachis oil used in the experiments described was kept in a cool dark place when not in use and no decomposition of the carotene was detected when the depth of colour of the solution after some months was measured in a Lovibond tintometer and compared with the colour of a freshly prepared solution.

Discussion.

These experiments support the general theory that in animals carotene behaves as a precursor of vitamin A as suggested originally by Moore [1929, 3]. Even with large doses of carotene the liver oils were only slightly more pigmented than when no carotene was given, but while the liver oils of the fowls fed on the vitamin A-free diet gave no blue colour with the antimony trichloride reagent, no absorption band at 325$\mu\mu$ and were biologically inactive, those of fowls given carotene in addition gave an intense blue colour (absorption band 610–630$\mu\mu$) with $SbCl_3$, an absorption band at 325$\mu\mu$ and proved adequate as a source of vitamin A when fed to rats.

The result of recent work on the relation of carotene to vitamin A makes it clear that biological tests alone cannot distinguish between carotene and the "classical" vitamin A and it would seem probable that vitamin A is a product of animal synthesis and ultimately owes its origin entirely to carotene. Land animals can obtain carotene from vegetable matter, while Ahmad [1930] has shown that carotene in diatoms is probably the source of the vitamin A of fish-liver oils.

Summary.

Chickens were successfully reared to maturity on a synthetic vitamin A-free diet to which either carotene or cod-liver oil concentrate was added. The carotene was not stored in the liver unchanged but was converted into vitamin A characterised by the blue colour given with $SbCl_3$ (absorption band 610–630$\mu\mu$) and the presence of an absorption band at 325$\mu\mu$.

The beaks and shanks of chickens, which had become colourless through the absence of carotenoids from the diet, did not become more yellow when carotene was added to it.

The poultry disease known as visceral gout would appear to be related to vitamin A deficiency and to be curable by the administration either of carotene or of cod-liver oil.

The vitamin A content of hen-liver oils is shown to be very high and the vitamin A requirements of the fowl large.

We have pleasure in expressing our thanks to Dr Moore for supplying the carotene used in the experiments, for carrying out the biological tests recorded

and for his unceasing interest in the work; to Imperial Chemical Industries, Ltd., for a grant which helped to defray the cost of the experiments; to Messrs Joseph Nathan, Ltd., for generously supplying the ostelin and cod-liver oil concentrate used, and to Miss A. C. Woods and Miss H. Kennedy of the Agricultural Research Institute of Northern Ireland at Hillsborough, without whose skilful care of the fowl the experiments would have been impossible.

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XXXIV. VITAMIN A AND CAROTENE.
VII. THE DISTRIBUTION OF VITAMIN A AND CAROTENE IN THE BODY OF THE RAT.

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In a preceding communication of this series [Moore, 1930] it was shown that the feeding of excess of carotene to albino rats resulted in an accumulation in the liver of large amounts of the colourless vitamin A, and the inference was drawn that the pigment, or some part of it, is the parent substance of the vitamin, capable of conversion in the normal course of metabolism. In this work attention was focused on the liver on account of its importance in the storage of the vitamin, and no attempt was made to examine other organs, or to decide whether the conversion of carotene is effected in the alimentary tract or after absorption into the blood stream. It seemed desirable, therefore, to make a detailed study of the distribution of vitamin A and carotene throughout the body and intestinal contents of rats receiving large amounts of carotene, in the hope of gaining further knowledge on these points.

Since Palmer and Kennedy [1920] have shown that carotene does not appear in the body fat of the rat even when large amounts are introduced into the diet, it is natural that the question of the distribution of the pigment in this animal should have hitherto attracted little attention. The question of vitamin A distribution, on the other hand, has been studied by numerous workers, notably Sherman and Boynton [1925], who used the rat-growth technique in estimating the vitamin, and by Kerppola [1930], who has employed the antimony trichloride reaction. Using tissues from rats deriving their supplies of vitamin A from the inclusion in their diet of $33\frac{1}{3}$ % of dried milk powder, the American workers found that daily doses of 0·02 g. liver, 0·1 g. lung, 0·1 g. kidney, or 4·0 g. of muscle were necessary to promote growth in other rats, and it was deduced that under these nutritional conditions nine-tenths of the vitamin A reserves of the body (neglecting those of the adipose tissue and skin) were to be found in the liver. The results of Kerppola, although not reduced to quantitative terms, suggest a similar distribution in regard to the liver and lungs. A positive reaction was also obtained from the intestines and contents, but negative results were given by all other organs examined. The predominance of the liver in the storage of the vitamin has

[1] In the whole time employment of the Medical Research Council.

also been reported in many other animals in addition to the rat; mention may be made of the work of Rosenheim and Webster [1927] on the fulmar petrel, and the comparison by Ahmad and Drummond [1930] of the vitamin A activities of the liver and body oils of fishes.

Experimental.

The experimental procedure was designed to determine the relative distributions of the pigment and vitamin under conditions in which the former was supplied in amounts greatly in excess of the immediate physiological requirements. Albino rats received synthetic diets in which excess of carotene was included, for reasons of convenience and economy, as red palm oil or, in one instance, as carrot-fat. After receiving these diets for periods sufficiently prolonged to ensure that a large superfluity of pigment had been made available certain of the rats were killed by coal gas and examined without further treatment. Others, with a view to the removal of traces of unaltered carotene that might have been present in the blood stream, were transferred to a diet deficient in vitamin A for appropriate periods before killing.

In estimating the amounts of vitamin A and carotene in the various tissues the methods employed were essentially those previously adopted in the extraction and colorimetric examination of liver oils [Moore, 1930]. Some modification, however, was necessary in the procedure adopted in the interpretation of results, since it was found that in red palm oil and carrot-fat the ratio of the natural yellow value to the blue value given in the antimony trichloride reaction was not more than about 4 to 1, as compared with the value of 11 to 1 previously quoted for pure carotene. Although at first sight this low ratio might be taken to imply the presence of carotene and vitamin A simultaneously, it is actually most improbable that this should have been the case, since no evidence of absorption at 610–630 $\mu\mu$, the position characteristic of vitamin A, could be detected when the blue colorations produced by these materials in the $SbCl_3$ reactions were examined spectroscopically. Two alternative explanations of the discrepancy may be put forward. In the first place since determinations of both yellow and blue values by the tintometer technique are subject to large experimental errors, the ratio between them becomes subject to a much greater error, so that the difference observed between the ratios may actually be less significant than it appears. Secondly it is probable that the yellow value of carotene, under certain natural conditions[1], may deteriorate in much the same way as it does under the action of benzoyl peroxide [Moore, 1929] leaving the blue value unchanged. In interpreting the present results confusion will be avoided by basing comparisons upon the yellow/blue ratios actually determined in the red palm oil and carrot-fat.

[1] Evidence of such deterioration has been obtained in the course of experiments in collaboration with Dr Woolf on the aerobic incubation of carotene with tissue preparations.

The distribution of vitamin A and carotene in rats receiving diets rich in carotene.

Rat 1, ♀ (*118 g.*). *Carrot-fat diet* (*results shown in Table I*). After receiving a diet deficient in vitamin A for 29 days this rat was cured by the administration of carrot-fat, admixed with the diet, at the level of about 250 mg.

Table I.

Organ	Wet weight (g.)	Weight of fat (g.)	Yellow units	Blue units	Inference
	Rat 1, ♀ (*118 g.*). *Carrot-fat diet.*				
Stomach	3·1	0·048	200	250	Carotene?
Small intestine and contents	3·5	0·055	100	100	Carotene?
Large intestine and contents	3·1	0·147	10,000	3,500	Carotene
Liver	9·1	0·063	280	2,500	Vitamin A
Brain	1·1	0·018	0	0	—
Heart	0·7	0·005	0	0	—
Kidneys	1·5	0·008	0	0	—
Lungs	1·4	0·0111	0	0	—
Pancreas	0·05	0·008	0	0	—
Spleen	0·7	0·0074	0	0	—
Suprarenals	0·046	0·006	0	0	—
Thymus	0·8	0·0054	0	0	—
Intraperitoneal fat	2·7	0·49	0	0	—
	Rat 2, ♂ (*370 g.*). *Red palm oil diet.*				
Alimentary tract and contents	13	1·05	2,500	375	Carotene
Liver	15	0·274	250	70,000	Vitamin A
Carcase	342	71·4	140	160	?
	Rat 3, ♀ (*197 g.*). *Red palm oil diet.*				
Small intestine and contents	6·2	0·1664	360	100	Carotene
Liver	10·2	0·1616	980	70,000	Vitamin A
Lungs	1·6	0·0268	0	15	Vitamin A
	Rat 4, ♀ (*208 g.*). *Red palm oil diet.*				
Liver	7·6	0·1395	570	80,000	Vitamin A
Blood	4·6	0·0195	1	0	—
Kidneys	2·2	0·0886	1	10	Vitamin A?
Lungs	1·3	0·0318	1	10	Vitamin A?
Intraperitoneal fat	11·4	9·84	20	150	Vitamin A

daily. The carotene content of the latter was equivalent to 40 natural yellow units and 15 $SbCl_3$ blue units per mg. The yellow/blue ratio in the ingested fat was therefore about 2·7, and the daily intake of carotene about 20 mg. Carotene feeding was continued for 37 days. Upon *post mortem* examination positive $SbCl_3$ reactions were given by the fats derived from the stomach, small intestine, large intestine, with their respective contents, and by the liver, negative reactions by the fats from the remaining organs and also by the intraperitoneal fat. In the fat from the large intestine a yellow/blue ratio of about 3 was given, suggestive of unchanged pigment, and this conclusion was supported by the observation of an absorption band at 590$\mu\mu$ in the $SbCl_3$ reaction. The fats from the stomach and large intestine showed a yellow/blue ratio of about unity, but since the characteristic band of vitamin A could not be detected in the $SbCl_3$ colorations it is probable that this low ratio

was essentially due to deterioration of the pigment, possibly on account of an abnormally long period of cold storage before examination. The liver oil contained a small amount of unconverted pigment, but the main chromogen present was vitamin A, as typified by a yellow/blue ratio of 0·1, together with absorption at 610–630 $\mu\mu$ in the $SbCl_3$ reaction.

It may be pointed out that the excess of carrot-fat administered to this rat was probably poorly tolerated, since the rate of growth at the time of killing was not rapid. The vitamin A content of the liver was not so high as in the cases described below, in which the feeding of a lower level of pigment was continued over longer periods.

Rat 2, ♂ (*370 g.*). *Red palm oil diet* (*Table I*). In the course of a separate experiment this rat had received for 119 days a synthetic diet in which vitamin A was included as 0·02 mg. of crystalline carotene daily; it was then transferred to a diet containing 15 % of red palm oil for a further period of 173 days.

The red palm oil employed had a natural yellow colour equivalent to 2·6 units per mg., and gave in the $SbCl_3$ reaction a value of 0·6 B.U. per mg. The yellow/blue ratio thus worked out at about 4.

For *post mortem* examination this rat was dissected into three portions: (1) the alimentary tract and contents, (2) the liver and (3) the rest of the carcase[1]. The fat obtained from the alimentary tract was characterised by a yellow/blue ratio of about 6, indicating the persistence of unconverted pigment. In the liver a very high level of vitamin A was found, characterised by a yellow/blue ratio of about 0·003 and by the appearance in the $SbCl_3$ test of the usual 610–630 $\mu\mu$ absorption band. The carcase fat, on the other hand, gave only a faintly positive reaction, so transient that it was impossible to make spectroscopic measurements. The yellow/blue ratio, moreover, may in this case have been deceptive, since it is probable that the yellow value observed was rendered unduly high through the admission of particles of diet adhering to the skin. All that can be said with safety, therefore, is that the colour value of this fat, whether attributable to vitamin A or carotene, was of a very low order in comparison with the values given by the fats of the liver and alimentary tract.

Rat 3, ♀ (*197 g.*). *Red palm oil diet* (*Table I*). The preparatory feeding of this rat was exactly similar to that adopted in the previous case, except that the red palm oil diet was given for the slightly longer period of 273 days. Only the small intestine, liver, and lungs were examined, positive $SbCl_3$ reactions being given in all three cases. In the intestine fat a yellow/blue ratio of about 3·6 was observed, suggesting the persistence of unchanged pigment. The usual high concentration of vitamin A together with a small amount of unconverted pigment was found in the liver. The lung fat gave only a faint reaction,

[1] Solution was effected quite easily without even mincing by warming with 5 % aqueous KOH in the usual way, although heating had naturally to be continued for a somewhat longer period than was necessary in the case of smaller amounts of tissues.

which, from the absence of accompanying yellow pigmentation, may probably be ascribed to the vitamin.

Biological tests. As a check on the genuine character of the extremely high colour values given by the liver oils throughout the present experiments it was decided in this case to carry out parallel rat-growth tests, the results of which are shown in Fig. 1. The liver oil was effective at 0·005 mg. (the lowest dosage examined), and thus possessed a biological activity in good agreement with its colour value.

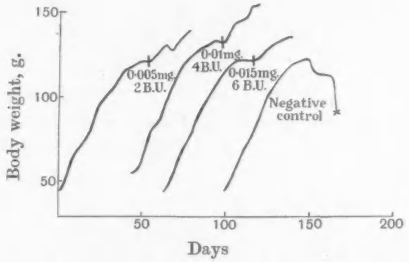

Fig. 1. Confirmation of the vitamin A activity of a representative rat-liver oil (that of rat 3, Table I) by rat-growth tests. The basal diet contained Glaxo caseinogen 20 %, rice starch 60 %, palm kernel oil 15 %, salt mixture 15 %, supplemented by 10 % of dried yeast and one drop of radiostol daily. The test doses were diluted in arachis oil and administered at the points indicated.

Rat 4, ♀ (208 g). Red palm oil diet (Table I). Preparatory feeding was again similar, the red palm oil diet being given for 278 days. The liver, kidneys, lungs, intraperitoneal fat and blood were examined, positive $SbCl_3$ reactions being given by all except the blood. The liver showed the usual high concentration of vitamin A, with small amounts of unconverted pigment. The kidneys, lungs and intraperitoneal fats all gave faintly positive reactions suggestive, in the absence of corresponding intense yellow coloration, of small amounts of vitamin A. The blood, which was obtained *post mortem* by cutting the blood vessels in the thoracic cavity just above the diaphragm, gave no indication of containing either carotene or vitamin A, although much of the sample taken must have flowed directly from the liver[1].

The distribution of carotene and vitamin A in rats restricted to a diet deficient in vitamin A after the preliminary feeding of excess of carotene.

Rat 5, ♂ (340 g.). Red palm oil diet, followed by vitamin A-free diet (Table II). Preliminary feeding was similar to that adopted in the preceding cases, the red palm oil diet being given for 176 days. Before killing, a diet deficient in vitamin A was given for a further period of 10 days.

On *post mortem* examination a faintly positive reaction probably due to

[1] The result would indicate great efficiency on the part of the liver in removing carotene from the general circulation of the rat, which is in good agreement with its power of regulating the vitamin A concentration (see discussion).

the incomplete removal of traces of red palm oil was given by the fat of the small intestine. The fats from the stomach and large intestine gave reddish reactions, which can probably be ascribed to the presence of arachis oil in the vitamin A-free diet. In the liver a high concentration of vitamin A persisted together with small amounts of unconverted pigment. Of the remaining tissues the muscle and peritoneal fats gave faintly positive reactions suggesting the presence of vitamin A at a low concentration, while in all other cases negative results were obtained.

Table II.

Organ	Wet weight (g.)	Weight of fat (g.)	Yellow units	Blue units	Inference
Rat 5, ♂ (340 g.). Red palm oil diet, followed by vitamin A-free diet.					
Stomach and contents	2·2	0·0508	0	Reddish	?
Small intestine and contents	5·7	0·145	20	12·5	Carotene?
Large intestine and contents	3·0	0·15	1	Reddish	?
Liver	15·5	0·208	150	50,000	Vitamin A
Brain	2·3	0·019	0	0	—
Heart	1·1	0·0075	0	0	—
Kidneys	3·0	0·032	0	0	—
Lungs	2·4	0·025	0	0	—
Pancreas	0·78	0·015	0	0	—
Spleen	0·55	0·004	0	0	—
Suprarenals	0·04	0·004	0	0	—
Testes and seminal vesicles	1·93	0·0669	0	0	—
Thymus	0·85	0·007	0	0	—
Thyroids and parathyroids	0·6	0·0064	0	0	—
Muscles	117	3·9	10	40	Vitamin A
Intraperitoneal fat	23	12·2	18	120	Vitamin A
Bones	55	1·6	5	Reddish	?
Skin	76	13·0	40	Reddish	?
Rat 6, ♂ (300 g.). Red palm oil diet, followed by vitamin A-free diet.					
Small intestine and contents	5·0	0·5127	4	Reddish	?
Liver	14·2	0·1978	15	38,000	Vitamin A
Intraperitoneal fat	13·0	10·7	8	80	Vitamin A?

Rat 6, ♂ (300 g.). Red palm oil diet, followed by vitamin A-free diet (Table II). Preliminary feeding treatment was exactly as in the preceding case, but the final period on the vitamin A-free diet was now increased to 68 days. Only the small intestine and contents, liver, and intraperitoneal fat were examined. In the case of the small intestine a negative result was obtained. In the liver the concentration of vitamin A still remained at a high level, but no appreciable amounts of unconverted pigment were present. The intraperitoneal fat still gave a reaction suggesting the presence of vitamin A in relatively low concentration.

The excretion of carotene in the faeces of rats receiving excess of carotene.

Two samples of faeces were collected from the combined droppings of the rats receiving red palm oil, after the animals had received this diet for 131 and 234 days respectively.

The results obtained in both samples were very similar (Table III). It should be noted that in each case the yellow/blue ratio was about 6, pointing

to the presence of unconverted pigment. The concentration of pigment attained actually rose to quite a high level, representing, from calculations based on the $SbCl_3$ colour value, some 4 % of the faeces fat, or a 12-fold concentration over that of the ingested palm oil.

Table III.

Wet weight (g.)	Weight of fat (g.)	Yellow units	Blue units	Inference
Sample of faeces collected after 131 days of red palm oil feeding.				
1·54	0·097	4,500	750	Carotene
Sample after 234 days.				
7·3	0·458	23,000	3500	Carotene

The second sample of faeces fat was tested biologically as a source of vitamin A to rats, and was found active at a level of 0·25 mg. daily, which corresponds, on a basis of the $SbCl_3$ value, to a dosage of 0·01 mg. of actual carotene. The growth curves are shown in Fig. 2.

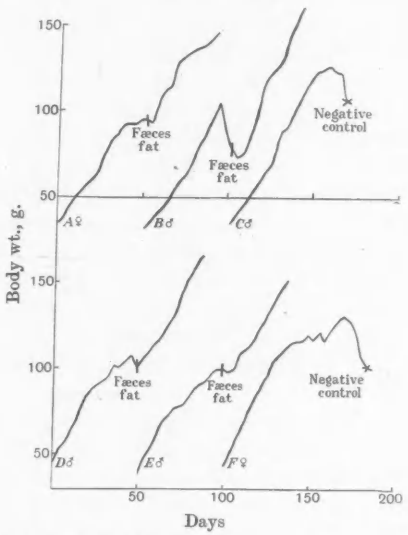

Fig. 2. The vitamin A activity of the faeces fat of rats receiving red palm oil. Rats *A*, *B* and *C* received a basal diet containing Glaxo caseinogen 20 %, rice starch 60 %, palm kernel oil 15 %, salt mixture 5 %, supplemented by 10 % of dried yeast and one drop of radiostol daily. Rats *D*, *E*, *F* received a diet differing only in the replacement of the palm kernel oil by additional rice starch. Doses of 0·25 mg. of faeces fat, equivalent to 0·01 mg. of carotene (1·8 B.U.) were administered in small amounts of arachis oil at the points indicated.

Discussion.

The site of the conversion of carotene. A most consistent feature of the above results is the persistence of carotene, apparently unchanged, within the alimentary tracts of those animals which had received diets rich in carotene

up to the time of killing. It is obvious, therefore, that conversion to the vitamin is not effected in the course of digestion, but at some period subsequent to absorption into the blood stream. Since the liver is unique not only in its ability to hold large stores of vitamin A, but also in containing noteworthy amounts of unchanged pigment it would seem unnecessary to look beyond this organ for the site of the conversion of carotene. Further indirect evidence by other workers may be quoted in support of this view. Rydbom [1930] has carried out experiments on the fate of carotene when injected intramuscularly into the legs of rats, and has recovered *post mortem* substantial amounts of the pigment in apparently unchanged state from the tissues surrounding the site of injection. This result would at least suggest that the power to convert carotene to vitamin A is not possessed indiscriminately by all animal tissues. The special importance of the liver, however, is most strikingly illustrated by the work of Buckley *et al.* [1930], who have encountered cases of parenchymatous degeneration of the liver in cattle, presumably produced by the eating of poisonous plants, in which this organ becomes so overcharged with carotene that it appears deep yellow in colour, and is capable of yielding large amounts of crystalline carotene on suitable treatment. Since in the present writer's experience normal ox-liver oils have invariably been found to contain large amounts of vitamin A, accompanied by relatively small amounts of carotene (so small as to render their isolation almost impossible by means at present available), the above condition presents a pathological picture of the failure of carotene conversion coincident with a degeneration of the liver tissues.

The capacity of the liver in vitamin A storage. Apart from suggesting the probable site of the conversion of carotene, the present experiments are of interest in demonstrating the extremely high levels to which the vitamin A reserves of the liver may rise when lavish amounts of carotene are included in the food supply. In the case of the rat these levels may attain values quite out of proportion to immediate or future requirements. Thus in rat No. 2 (Table I) the liver was found to contain vitamin A equivalent to 70,000 blue units in the $SbCl_3$ test. If we assume that 2 B.U. per day would have represented the minimal requirements of this animal, then this store would have been adequate to have lasted for about a century, as compared with the natural life period of about 3 years.

Vitamin A in the remainder of the body. In contrast with the high level attained in the liver the amounts of vitamin A distributed throughout the remainder of the body are relatively small, although in some cases representing a supply sufficient for two or three months. In confirmation of Sherman and Boynton [1925] indications of the presence of vitamin A were observed in the fats obtained from the lungs and kidneys of certain animals (Nos. 3 and 4, Table I). The colour values obtained agree well with the minimal rat doses of the fresh tissues as determined by these workers, although, of course, the conditions of vitamin A excess were quite different. It must remain doubtful, however, whether these results should be taken to imply

that the organs concerned play any outstanding part in the metabolism of the vitamin. Not only were negative results given by the same organs in two other rats (No. 1, Table I and No. 5, Table II) but even in the cases under review the superiority of the vitamin A content over the general level in the stored fat of the body was so small as to be of very doubtful significance. Indeed from the aspect of quantity as opposed to concentration the stored fat (*e.g.* intraperitoneal fat) certainly contained the great bulk (at least 90 %) of the total vitamin A reserves found outside the liver. The consistently negative results given by the remaining organs of rats Nos. 1 and 5, while not necessarily implying that vitamin A was completely absent, must similarly suggest that the concentration of vitamin did not rise above that of the stored fat, which would not have given appreciably positive results if only minute quantities similar to those obtained from the various organs, had been available for testing.

The rôle of the liver in the regulation of the distribution of vitamin A. When an attempt is made to co-ordinate the conclusions reached in the two preceding paragraphs it appears that the liver must play a large part in the regulation of the concentration of vitamin A throughout the remainder of the body. This rôle, of course, is linked up with that of storage, but whereas the function of the liver in receiving and concentrating superfluous amounts of vitamin A from the diet has always been fully realised, its complementary function in facilitating the maintenance of a low level of vitamin A concentration in the body tissues has not been sufficiently appreciated. The case of rat No. 2 (Table I) provides a good illustration of the importance of this function. The concentration of carotene in the red palm oil included in the diet of this rat was equivalent to about 0·6 B.U. per mg., while in the liver oil the concentration of vitamin A was equivalent to about 250 B.U. per mg. If, for the sake of simplicity in argument, we compare these values directly, without making any allowance for the fact that different chromogens are involved, then the concentration of vitamin A in the liver oil may be considered to correspond roughly to an activity some 400 times greater than that of the ingested red palm oil. When on the other hand we turn to the fat derived from the remainder of the body (carcase fat) we find that the concentration of vitamin A is equivalent to not more than 0·002 B.U. per mg., which corresponds to only 1/300 of the activity in the ingested fat, or less than 1/100,000 of the activity of the liver oil. From these results it may be inferred that, while the vitamin A concentration in the liver may vary over an enormously wide range without producing any obvious effect, the concentration in the remainder of the body does not rise above a prescribed limit.

The efficiency of the conversion of carotene. In the previous communication [Moore, 1930] a point of difficulty arose in explaining how cod-liver oil concentrates, supposed by Drummond and Baker [1929] to contain only a minute proportion of actual vitamin A, could approach pure carotene so closely in

vitamin A activity. It was suggested either that the estimate of Drummond and Baker might be unduly low, or alternatively that the conversion of carotene might be of an inefficient character, thus necessitating its administration at a dosage greatly exceeding the amount actually converted into the vitamin.

It may be recalled that carotene gave a value in the $SbCl_3$ reaction of about 180 B.U. (at 590 $\mu\mu$), and was effective in growth tests at levels down to about 0·004 mg. A typical cod-liver oil concentrate, on the other hand, gave a colour value of about 270 B.U. (at 610–630 $\mu\mu$) and was found in one instance to be biologically active at 0·0033 mg., although 0·01 mg. was necessary to ensure regular results. These figures indicated that vitamin A must at least have a slightly higher colour value than carotene, but since it was by no means necessary to suppose that the different blue colorations given by carotene and vitamin A bore exactly the same relation to biological activity, this evidence did not seem incompatible with the simple assumption that the conversion of carotene to the vitamin might be almost complete, and that vitamin A might after all represent the main constituent of the cod-liver oil concentrates.

The data obtained in the present experiments, however, must now lead to a revision of this view, since in several cases the rat-liver oils themselves (not their unsaponifiable fractions) gave colour values much higher than those previously determined for cod-liver oil concentrates[1]. Thus the liver oil of rats Nos. 3 and 4 (Table I) gave colour values of about 430 and 600 B.U. per mg. respectively. Preliminary experiments have indicated that the proportion of unsaponifiable matter in these oils is much greater than in cod-liver oils, but it is safe to assume that by saponification and the removal of sterols, etc. the colour values could be easily raised to a much higher level. Since there is every reason to believe that these colour values bear a genuine relation to the biological values of the oils (see Fig. 1) it must be inferred that preformed vitamin A not only possesses a much higher colour value than carotene, but also, under the usual conditions of administration, is biologically effective in much smaller doses.

Two alternative explanations of these findings might be advanced. In the first case it might be suggested that carotene is heterogeneous, containing in small amounts a component responsible for its biological and chromogenic activity, which is concentrated and converted to vitamin A in the liver. Secondly it might be supposed that the absorption and conversion of carotene is normally inefficient, but that actual conversion, when attained, is accompanied by a great increase in chromogenic value. The converted portion of the pigment, as vitamin A, now becomes available for utilisation by a second animal without a similar heavy loss, and the minimal dosage is therefore correspondingly reduced. This latter alternative is supported by the appearance

[1] The writer has been privileged to examine chicken-liver oils of approximately equal activity prepared by Mr N. S. Capper.

of excess of unchanged carotene in the faeces of the rats used in the present experiments, and for the present is perhaps to be preferred.

The effect of dietary vitamin A deficiency upon pre-existing reserves of vitamin A and carotene. The results obtained in the cases of rats Nos. 5 and 6 (Table II) are of interest in showing the effect of exposure to a diet deficient in vitamin A upon the reserves of vitamin A and carotene stored up during the period of excess. After receiving the deficient diet for periods of 10 and 68 days the vitamin A content of the liver oils remained extremely high. Similarly the intraperitoneal fats gave colour values differing but little from those shown by rats receiving carotene up to the time of killing. The most noteworthy change observed was the virtual disappearance of unchanged pigment from the liver oil of rat No. 6. The extremely low yellow/blue ratio (0·0004) shown by this oil supports almost to the degree of certainty the current view that pure vitamin A is completely colourless.

Summary.

1. Albino rats were given diets containing lavish amounts of carotene either as red palm oil or carrot-fat for prolonged periods. The animals were then killed, and estimations of vitamin A and carotene were carried out on the dissected tissues by colorimetric methods.

2. Excess of carotene was found to persist apparently unchanged throughout the alimentary tract. The pigmented fat derived from the faeces was found to be biologically active at a level based upon its apparent carotene content.

3. The liver oils invariably contained vitamin A at extremely high concentrations, the oils themselves in several cases giving higher colour values than typical cod-liver oil concentrates. Small amounts of unconverted pigment were also present in the liver oils of all rats which had received carotene up to the time of killing.

4. Indications of the presence of vitamin A were usually shown by the "storage" fats of the body, the concentration per unit of fat, however, being not more than about 1/100,000 of the concentration found in the liver oils. Similar indications were also given by the lung and kidney oils of certain rats, but in general negative results were obtained in all organs other than the liver. A single test upon blood also gave a negative result.

5. Exposure of the rat to dietary vitamin A deficiency subsequent to carotene feeding led to no dramatic departure from the above distribution, except that after 68 days of such treatment unchanged pigment had virtually disappeared from the liver oil.

6. From the above evidence it is deduced that the conversion of carotene to vitamin A probably takes place in the liver, that the efficiency of the conversion is by no means quantitative and that the liver plays an important rôle in the regulation of the concentration of the vitamin throughout the remainder of the body.

My thanks are due to Dr L. J. Harris for his valuable criticism, and to Mr K. MacLennan of Lever Brothers, Ltd. for supplies of red palm oil. The care of the experimental animals was in the reliable hands of Mr A. Ward.

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XXXV. THE BIOCHEMISTRY OF ALUMINIUM.
II. EXCRETION AND ABSORPTION OF ALUMINIUM IN THE RAT.

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It has previously been shown by balance experiments with pigs that practically the whole of the aluminium provided as a supplement to the diet is voided in the faeces [Mackenzie, 1930]. With such animals there is a difficulty in devising a ration free from aluminium and accordingly this work has been supplemented by experiments with rats, where the ration could be built up by the use of highly purified foodstuffs.

The experiments of Flinn and Inouye [1928] on the excretion of aluminium by rats led them to believe that, when an aluminium salt was administered in the drinking water, 30 % of the aluminium was excreted in the urine and 70 % in the faeces. As details of the procedure are not given, it is uncertain whether contamination of urine by the faeces was prevented, and whether the drinking water was guarded against mechanical dissipation. The absorption of aluminium by rats, fed on diets containing aluminium over considerable periods, has been reported upon by Myers and Mull [1928]. There appeared to be some increase in the aluminium content of the tissues, though the method of analysis was not sensitive enough to give definite results. Wu [1929] states that rats fed on aluminised diets show an increased content of aluminium in the liver as compared with normal rats (0·97 mg. per 100 g. as compared with 0·59 mg. per 100 g.). References to experiments with other species were given in the previous publication [Mackenzie, 1930].

Experimental.

The chief difficulty in these experiments was to separate the three materials likely to contain aluminium, namely, the food, the urine and the faeces, as thoroughly as possible, while not interfering with the regular life of the animals. After a few trials the following method was adopted as the simplest and most satisfactory. The rats were kept in individual glass jars (15″ long, 11″ wide, 5″ high) with a glass plate closing the mouth (fixed in position by wire), but leaving a gap of ½″ at each side for air circulation. After preliminary cleaning the base of the jar was covered with a pad of filter-paper, made by folding a large sheet (40″ × 30″) into two or three layers of suitable shape,

on which was superimposed a fine iron wire gauze mat mounted on a stout wire frame which fitted the bottom of the jar accurately. The food container was of special construction designed to prevent scattering of food; its dimensions and a section are given in Fig. 1. The lid was held in position by bayonet catches, and through the hole in its centre the rat could remove food by the mouth, but owing to the sloping guard ring was unable to drag it out with its paws. Water was supplied from drop-bottles, wired into position in the apertures at each side of the cage. The cages were cleaned out daily, the rat being removed to another cage; the faeces were brushed into a wide-mouth bottle and the gauze mat was taken out and shaken to remove any small particles of food, which were collected and returned to the food trough.

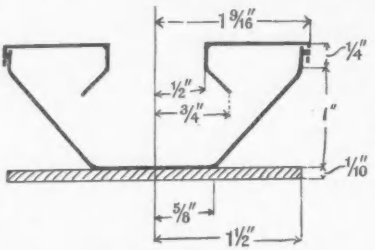

Fig. 1. Diagram of food trough. Body of 12-gauge zinc sheet. Base of hard lead sheet.

The rats used in these experiments were members of larger groups of 30–40 animals which were employed on feeding and fertility tests; these groups of rats were chosen from a number of young albino or Norwegian rats recently weaned, which were divided into two symmetrical groups, one receiving a synthetic diet approximately free from aluminium and adequate for growth, the other a similar diet with the addition of 0·1 % aluminium. Growth was usually regular, the animals gaining in weight steadily from 40 g. to 200 g. in the course of 12–16 weeks. In the first two metabolic experiments, fully-grown animals were used, three animals on the control, and three on the aluminised diet, and each animal occupied a separate cage. In the third experiment, animals 12 weeks old, averaging 120 g. in weight were employed, and the three animals of each group were placed in the same cage. It was hoped by this means to reduce the amount of aluminium which could not be accounted for, but the improvement in the result was not noticeable.

Analysis of material.

The food. Sufficient amounts of the control diet and aluminised diet were removed from the main bulk of the stock rations, well mixed, and sampled. The control diet was analysed by the spectrographic method (a modified version of the method used by McCollum, Rask and Becker [1928]) and the aluminised diet by the method of Schmidt and Hoagland [1912].

The faeces. These were collected daily during the metabolic period and at its conclusion were dried, ground and analysed, the control group by the colorimetric method [Myers and Morrison, 1928], the aluminised group by the gravimetric method.

The urine. The filter papers were removed from the cages, dried, brushed

lightly to remove any traces of adherent food, ashed, and analysed by the colorimetric method. Blank determinations were made on a few fresh filter papers.

Table I. *Diets.*

	Exps. 1 and 2		Exp. 3	
	Control	Aluminised	Control	Aluminised
Yeast	25 parts	25 parts	24 parts	24 parts
Caseinogen	90 ,,	90 ,,	88 ,,	88 ,,
Salt mixture 185	20 ,,	20 ,,	20 ,,	20 ,,
Cellulose	10 ,,	10 ,,	10 ,,	10 ,,
Butter	140 ,,	140 ,,	80 ,,	80 ,,
Starch	215 ,,	195 ,,	215 ,,	200 ,,
A.P.B.P.*	—	20 ,,	—	15 ,,
Al. content mg./100 g.	0·11	106·5	0·11	85·4

* This represents a commercial brand of baking powder, containing acid calcium phosphate and sodium aluminium sulphate; it was decomposed with water and dried, the product containing about 3 % of aluminium.

Table II. *Results of experiments carried out on groups of three rats.*

	Exp. 1		Exp. 2		Exp. 3	
	Control	Aluminium	Control	Aluminium	Control	Aluminium
Food eaten (g.)	540	540	362	393	356	321
Duration (days)	12	12	7	7	9	9
Food eaten per rat daily	15	15	17·2	18·7	13·2	11·9
Total aluminium ingested (mg.)	0·59	575·0	0·395	418·5	0·39	274·1
Aluminium output (mg.):						
(*a*) in urine	0·006*	0·026*	0·006*	0·014*	0·008	0·024
(*b*) in faeces	0·47	484·2	0·29	394·1	0·44	261·3
Total aluminium output (mg.)	0·476	484·2	0·296	394·1	0·448	261·3
Output as % ingestion	80·7	84·2	74·9	94·2	114·9	95·4

* Total of three determinations.

Urinary excretion. In arriving at the figure for urinary excretion it is necessary to take into account the value of the blank as determined on several fresh sheets of filter-paper. The average of six blanks was 0·003 mg., and the largest single figure obtained was 0·008 mg. Since the analytical method employed is of doubtful accuracy with amounts of aluminium less than 0·005 mg., only two of the results for urinary excretion can be accepted as definitely indicating the presence of aluminium—the "aluminium groups" of Exps. 1 and 3. In these cases, it is possible that a small amount of the food employed in the experiment may have passed through the gauze mat and become incorporated in the paper when analysed; an amount of 20 mg. so included would be sufficient to account for the amount of aluminium observed. But even if this is not so, the amount of aluminium is so small in relation to the total ingestion as to make it of little significance.

The faeces. (*a*) *Recovery of aluminium ingested by control animals.* The disparity in the figures of intake and output is probably due to analytical error. The amount of aluminium is in all cases very small, and the systematic error

is consequently much greater than usual. Combination of all the results for the control animals gives an average recovery of 90·2 %, or an average possible absorption of 0·04 mg. per rat.

(*b*) *Recovery of aluminium ingested by aluminium-fed animals.* In the second and third experiments a high level of recovery, approximately 95 %, was obtained, but the recovery in the first experiment was unexpectedly low. This was assumed to be due to coprophagy, though this had not been observed during the experiment; it may have occurred, since the rats were supplied with a fixed amount of food daily, and it is possible that this was not sufficient in the case of one or two rats. In the subsequent experiments, an excess of food was always supplied, and only the total weight eaten for the whole period was ascertained. The recovery of aluminium was approximately equal in the two latter cases, and it seems doubtful whether attention to detail could reduce the unavoidable loss much further. Two causes may operate to cause error: mechanical losses, and variation in the rate at which the animals consume their food. It seems unlikely that the latter can be a serious source of error. The use of three animals over a period of 7 days would reduce such variation to a very small percentage of the total.

Possible absorption of aluminium as determined by analyses of the animal organs.

In the course of the feeding and rearing experiments with which the above metabolic experiments were connected, a number of the adult rats were killed and their internal organs were analysed for aluminium. The results were considered applicable to the problem immediately under consideration, as these rats received exactly the same food and treatment throughout their life-period. The analyses were conducted on the organs removed from 8–10 rats, so as to minimise systematic errors, experience having shown that with the smaller amount obtained from 3 rats these were apt to be unduly large.

The analytical data for two groups of rats are given in Table III. Series A contained the rats used in Exps. 1 and 2. Series B contained the animals used in Exp. 3. All rats were full-grown and healthy, with an average weight in excess of 200 g.

Table III.

	No. of rats examined	Aluminium found in mg./100 g. fresh material				
		Liver	Lung	Heart	Stomach	Small intestine
Series A:						
Control diet	10	1·8	0·32	—	3·8	2·8
Aluminium diet	8	2·2	0·22	—	1·1	2·9
Series B:						
Control diet	9	1·6	0·16	0·20	1·35	1·15
Aluminium diet	8	1·2	0·14	0·24	2·6	2·2

These results cannot be considered as evidence that absorption of aluminium occurs through ingestion of aluminised food; the variations between the

different groups are not greater than have been obtained in other series of analyses in which no aluminium was fed. The general trend of these experiments is to show that aluminium plays no important part in the metabolism of the rat, and supports the theory that aluminium, when ingested, is quantitatively excreted through the alimentary tract.

Summary.

Rats receiving a diet containing aluminium excrete the aluminium entirely by way of the alimentary tract, and there is no clear evidence that any of the aluminium is excreted in the urine.

No definite evidence of absorption of aluminium by the internal organs of such rats has been established.

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XXXVI. CALCIFICATION OF THE BONES OF RATS ON A DIET LOW IN ERGOSTEROL.

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The statement is frequently made that ergosterol is a substance which cannot be made in the animal body but there does not seem to be any real evidence that this is the case.

Beumer [1927] attempted to trace the fate of ergosterol ingested by an infant; of 0·4 g. ingested, he only succeeded in recovering 0·147 g. from the stools, but the observation does not shed much light on the problem.

In contrast with earlier workers both Channon [1925] and Randles and Knudson [1925] concluded that the rat can synthesise cholesterol. One of the chief difficulties in such an experiment is to supply the fat-soluble vitamins to the experimental animals free from sterols. Channon met this difficulty by using the unsaponifiable matter from cod-liver oil, after having first freed it from cholesterol by precipitation with digitonin; such a procedure seems satisfactory and would presumably leave the rat supplied with vitamins A and D. Randles and Knudson used dried alfalfa leaves, which had been extracted with cold ether; the method was held to remove the sterols from the leaves, while not extracting the vitamin A; the extraction of the sterols by this method could however hardly be regarded as complete and the experiment would seem to lack conclusiveness on this ground.

Even if cholesterol can be synthesised in the rat's body, as Channon's work indicates, it cannot therefore be concluded that this is also true of ergosterol. It seemed possible that the problem might be attacked indirectly, not by an attempt to estimate the ergosterol balance but by ascertaining whether the administration of diets, rich in ergosterol on the one hand, and rendered as deficient as possible in it on the other, had any influence on calcification in rats irradiated with ultra-violet light and deprived of any source of vitamin D through the mouth.

It was obvious that the experiment must be a long one, in order to aim at a depletion of any reserves of ergosterol in the rat; the diet would therefore have to be as complete as possible, so that prolonged well-being might be assured, while it must at the same time be rendered as free as possible from vitamin D and from sterols. The chief problem lay in the preparation of sources of vitamins A and B which should fulfil this requirement.

The experiment lasted about 4 months and was therefore a prolonged observation on the behaviour of rats on a fat-free diet. Certain observations were made in the course of it, on the occurrence of a condition of scaliness of the tail, described in particular by Burr and Burr [1929], as occurring on diets free from fat. These observations are included in an ensuing note.

Experimental.

Preparation of the diet. The diet was composed as follows:

Caseinogen	...	20
Wheat starch	...	65
Salt mixture	...	5 (McCollum 185 [McCollum and Davis, 1914][1])

Daily supplements were given separately of a vitamin A concentrate equivalent to 1·0 g. of the original spinach, and of a vitamin B concentrate equivalent to 1·0 g. of dried yeast.

The caseinogen used was that of the British Drug Houses, "fat- and vitamin-free," which is extracted with alcohol and ether.

The wheat starch received one extraction with cold light petroleum, by which means a small amount of a yellow oil was removed.

The caseinogen, starch and salt mixture were mixed with freshly distilled water and steamed in a double saucepan. By this means the starch grains were burst and any risk of refection obviated [see Roscoe, 1927, 1]. The mechanism of refection is not understood, but if, as seems possible, it depends on the elaboration of the B vitamins by some organism within the intestinal tract and their subsequent absorption by the rat, it would also appear possible that such organisms, particularly if they were yeasts, might also elaborate ergosterol and provide it to the rat. It was advisable therefore to take special precautions against refection.

An extract from spinach leaves was prepared to be a source of vitamin A. It was hoped that this when rendered sterol-free would supply a source of vitamin A which would also be free from vitamin D. Willimott and Wokes [1927] found that an ether extract of spinach, when fed in the equivalent of 5·0 g. of fresh spinach daily, supplied adequate vitamin A and had no significant influence on calcification, but Chick and Roscoe [1926] and Roscoe [1927, 2] found that the fresh leaves of summer spinach did exercise a small but definite antirachitic effect. It is certainly possible to render spinach strongly antirachitic by artificial ultra-violet irradiation, but the amount of vitamin D appears to be negligible unless the spinach is exposed to special

[1] Composition of salt mixture:

Sodium chloride	51·9
Magnesium sulphate	164·0
Sodium dihydrogen phosphate	104·1
Dipotassium hydrogen phosphate	286·2
Calcium phosphate	162·0
Calcium lactate	390·0
Ferric citrate	35·4

conditions of irradiation or insolation. At any rate the ether extract of a green leaf appeared to offer the best hope of providing a source of vitamin A, devoid of vitamin D. In the present state of knowledge, it is easy to see that it would have been better to have used recrystallised carotene as the source of vitamin A, since Moore [1929] has shown that purified carotene has no antirachitic activity. At the time, however, when the present experiment was carried out, it seemed unwise to proceed further in fractionation than to prepare a light petroleum extract of spinach, to saponify it and to use what was left of the unsaponifiable fraction after the sterols had been precipitated with digitonin.

Preparation of the extract of vitamin A. Two batches of extract were prepared. One was derived from 1500 g. of fresh prickly-seeded spinach (*Spinacia oleracea*) gathered in May. The product was used for a preliminary test to establish the vitamin A value of the extract. The second batch of 4200 g. of similar material was gathered at the end of September and beginning of October and the product was used for the main experiment.

The leaves were sorted and the larger pieces of stalk were removed. The leaves were dipped in boiling water and dried in front of a fan at a temperature of 37°. The dried leaves were powdered and sieved and extracted repeatedly with light petroleum (B.P. 40–60°), the solvent being subsequently distilled off at about 50°. From 1500 g. of fresh spinach about 2·6 g. of solid extract were obtained and from 4200 g. about 9·6 g. The extract was taken up with ether.

Saponification was carried out with sodium ethoxide, freshly prepared from metallic sodium and ethyl alcohol. Part of the alcohol was evaporated and water was added to the remaining solution, which was repeatedly shaken with fresh amounts of ether until the ether fraction ceased to show more than a little coloration. The ether was distilled off from the collected extracts, and after drying the yield of unsaponifiable matter was, from 1500 g. of spinach, 1·95 g., and from 4200 g., 3·8 g.

The unsaponifiable fraction was treated with digitonin in alcoholic solution. The solution was filtered to free it from the insoluble digitonide, which was washed with ether and alcohol. The alcohol was taken off and the residue was then taken up with ether and filtered to free it from the ether-insoluble excess of digitonin. When the ether was finally removed, a deep orange-coloured fraction was left, amounting to 0·92 g. from 1500 g. of spinach and 3·06 g. from 4200 g. of spinach.

The first batch of material, derived from 1500 g. of fresh spinach was taken up in hardened cottonseed oil and was fed to rats after a depletion period on a diet deficient in vitamin A. An amount corresponding with 1·0 g. of fresh spinach daily was found to supply a sufficient source of vitamin A over an extended experimental period of 70 days. The writers have found 0·02 and 0·03 g. of fresh spinach to supply adequate vitamin A for maintenance in the rat for an experimental period of 35 days [Hume and Smith, 1930].

The second batch of material, derived from 4200 g. of fresh spinach, was used for the sterol-free experiment. For this purpose the material was taken up with liquid paraffin (Internol of Messrs Allen and Hanbury); the preparation was stored in a brown bottle at about 0°; in these circumstances the yellow colour was well maintained and the preparation retained potency over a long period. It was so made up that one drop of the liquid paraffin contained the equivalent of 1·0 g. of fresh spinach; each rat received one drop daily of the preparation throughout the whole period of the experiment.

Preparation of the extract of the B vitamins. The concentrate of B vitamins was prepared from brewer's yeast by the method described by Chick and Roscoe [1929].

The yeast was washed four or five times with ice-cold water and pressed. About 15 kg. of such moist yeast were thrown into about 30 litres of boiling distilled water containing 0·01 % of acetic acid. The whole was again brought to the boil and filtered through Büchner funnels while hot. The clear filtrate was concentrated to a convenient small bulk, acidulated with sulphuric acid to about p_H 3·0 and stored at 0°. It was diluted and filtered again just before use.

The preparation was tested on rats, kept on open wire screens and fed on a diet deficient in the B vitamins, *i.e.* caseinogen (British Drug Houses, "fat- and vitamin-free") 300, wheat starch 750, hardened cottonseed oil 225, salt mixture (McCollum 185) 75, cod-liver oil 3–5 drops per rat daily. The diet was mixed with water and steamed. On an equivalent of about 1·0 g. of dried yeast daily, young rats grew well for an experimental period of 35 days. A similar dose was used throughout the main experiment. No source of vitamin C was given.

For a part of the animals ergosterol was added to the diet and for that purpose ergosterol supplied by the British Drug Houses was used. It was dissolved in "Internol" so that one drop contained $\frac{1}{10}$ mg. In this concentration it showed a tendency to crystallise out so that the bottle had always to be shaken before use.

Method of experiment.

The rats used were of the Lister Institute black and white strain; they were about 33–39 g. in weight and about 20–23 days old. They were kept singly in cages on open wire grids of mesh 3 squares to the inch to prevent consumption of faeces. The cages were washed several times a week, if necessary; it was feared that even a small contamination with faeces might supply ergosterol to the rats, through micro-organisms in the faeces.

Four litters of rats were used and were distributed evenly as regards sex and litter amongst four experimental groups; each of the four groups included five individuals, which were identical in sex and litter for each group. For the 1st week of experiment all four groups were treated alike; after that two of the groups received a daily addition of one drop of liquid paraffin, containing 0·01 mg. of ergosterol, while the other two groups received the same amount of liquid paraffin only. The experiment lasted 120 days, except in the case of one animal in each group (one litter), where it lasted 112 days.

About the 80th day of experiment it was hoped that the rats' ergosterol reserve, in those groups not receiving ergosterol, might be exhausted. Irradiation with a mercury vapour quartz lamp (Hewittic Electric Co.) was therefore instituted for one of the two groups receiving ergosterol and for one of the two not receiving ergosterol. The four groups therefore were receiving treatment as follows:

Group 1.	No irradiation	No ergosterol
Group 2.	No irradiation	Ergosterol
Group 3.	Irradiation	No ergosterol
Group 4.	Irradiation	Ergosterol

Irradiation was for 10 minutes every weekday at a distance of about 60 cm. It was found necessary to shield the eyes of the rats from time to time,

whenever they became sore from the irradiation, otherwise the animals ceased to thrive.

The animals were weighed at regular intervals. At the end of the experiment, all were killed and the femur and tibia of both legs were removed. These were dried, extracted with ether and alcohol and the ash content was determined.

Results.

The rats did well throughout the experiment and all survived the full period. The eyes of those which had not been irradiated were all normal or nearly normal at the end of the experiment; the condition of the eyes in those which had been irradiated could not be judged as regards xerosis, since, in spite of being frequently shielded, the eyes of these rats were rendered abnormal by the action of the mercury vapour lamp. Only three out of the twenty animals developed "snuffles" and only two showed small patches of congestion in the lungs at autopsy. The vitamin A supply throughout the experiment would therefore appear to have been quite adequate.

All the four rats belonging to one litter (No. 1228) developed small sore patches on the skin about the face and shoulders, towards the close of the experiment. This condition has often been observed before by the writers, in rats on other diets; it appears to be caused by an abnormal multiplication of lice, which cause irritation to the rat; the writers are inclined to correlate it with an unknown dietary deficiency.

All the rats developed some signs of the "scaly tail" condition, described by Burr and Burr [1929]. The tails were excoriated, shiny, annulated and scaly in parts and in some instances there were signs of the same condition on the skin of the feet. It would be natural to conclude with Burr and Burr that the condition was due to the absence of fat from the dietary, were it not that the same condition developed in the rats which were used for testing the material employed as a source of vitamin B. These latter rats received about 16 % of hardened cottonseed oil in their diet, together with 3–5 drops of cod-liver oil per head daily. The presence or absence of fat could not therefore have been the factor governing the development of scaly tail. The factors differentiating these experiments from others which the writers have carried out, and in which the condition has not been seen, would appear to be the type of caseinogen, the source of the B vitamins and the type of cage, with open wire screens to prevent coprophagy. A further investigation and discussion of the problem is included in the following paper.

Growth was strong at the start, being at first fully normal but after about 50 days, in most cases, it tended to slacken off; females had then reached a weight of about 150 g. and males of 200 g. An increase in the supply of B vitamins towards the close of the experiment did little to restore the growth rate and it is probable that the slackening was due to the lack of an unknown dietary factor, either that one, the lack of which caused the scaly tail condition, or another. A little acceleration of the growth rate took place after

the institution of irradiation about the 80th day of experiment in Groups 3 and 4, but if growth was being limited in any case by the lack of some other factor, any benefit to growth from the administration of vitamin D would be thereby obscured. The total growth response in the four groups, in the period after the institution of irradiation in Groups 3 and 4, was as follows:

Group 1.	No irradiation, no ergosterol	106 g.
Group 2.	No irradiation, ergosterol	112 g.
Group 3.	Irradiation, no ergosterol	129 g.
Group 4.	Irradiation, ergosterol	147 g.

The figures appear to show a small but distinct superiority in growth in the two groups which were irradiated.

Table I. *Percentage ash in the dried extracted bones of rats fed on a diet made as deficient as possible in ergosterol (Group 1), to which ergosterol was added (Group 2), which were irradiated with ultra-violet light for the latter part of the experiment (Group 3) and which were both irradiated and received added ergosterol (Group 4).*

Litter No.	Sex	No irradiation and no ergosterol	No irradiation and ergosterol	Irradiation and no ergosterol	Irradiation and ergosterol
1228	♀♀♂♂♂	60·6	62·4	61·9	62·4
1227		59·0	59·2	60·7	60·4
1255		60·3	61·0	61·9	61·8
1255		60·8	61·7	62·3	61·7
1272		59·8	61·2	61·5	61·0
	Average	60·1	61·1	61·7	61·5

The percentage ash in the dried extracted bones is set out in Table I. It is seen at once that all bones, even those in the negative control, Group 1, without ergosterol or irradiation, show a very high measure of calcification. In comparison with these, however, the two irradiated groups show a small but definite superiority in calcification; the range in each group is so small and the superiority of each individual irradiated rat over its corresponding litter mate in the negative control group is so regular that it would appear permissible to accept the result, in spite of the relatively high calcification in all groups. There is no difference between the calcifications of the two irradiated groups, indicating that abundance or deficiency of ergosterol in the diet made no difference to the calcification when the rats were exposed to ultra-violet light from a powerful artificial source. The second group, those rats which received ergosterol without irradiation, show a curious result. The individual values are more scattered and, while on the whole, barely inferior to the values in the irradiated groups, are definitely superior to those in the negative control group. The result seems to suggest that a rich supply of ergosterol in the diet promotes calcification when the only source of ultra-violet light is diffuse daylight in a North room. It must not be forgotten that ergosterol feeding in Groups 2 and 4 took place over the whole period of the experiment.

The whole experiment is marred by the high calcification of the negative controls, which is not easy of explanation. The salt mixture used is certainly not one on which a low calcification would be expected, but past experience suggests that some other explanation is needed. The reserves of the rats in vitamin D might have been high, but this has not been found to be the case when similar rats from the Lister Institute stock have been fed on rickets-producing diets. The only other explanation would seem to be that the vitamin A concentrate, prepared from spinach, also contained vitamin D; that possibility has already been discussed and dismissed as improbable, particularly in view of the smallness of the dose used, which was the equivalent of 1·0 g. of fresh spinach.

The result suggests that limitation of ergosterol in the diet did not act as a limiting factor in calcification when the rats were powerfully irradiated with ultra-violet light. It is not however possible to deduce from this result whether ergosterol can be synthesised in the rat's body or not; that some measure of synthesis takes place is one possible interpretation, but ignorance as to the rat's possible reserve of ergosterol and uncertainty as to whether the diet was completely devoid of, or only relatively deficient in, ergosterol, make it impossible to draw a definite conclusion, the results being such as they are.

If synthesis did take place, addition of ergosterol to the diet was still able to raise the calcification to a slightly higher level, when the supply of ultra-violet light was only very small.

Summary.

1. Experiments are described in which rats were fed on a diet made as free as possible from ergosterol, with the object of ascertaining to what extent this sterol can be manufactured in the animal body. Other rats were fed on the same diet with an abundant addition of ergosterol. After about 80 days half the rats in each series were irradiated with a mercury vapour quartz lamp daily, for about 40 days. At the end of the experiment the percentage ash in the dried fat-free bones of all the rats was determined.

2. The bones of all the rats showed a high percentage of ash. That in the bones of the irradiated rats receiving ergosterol was the same as in those of the irradiated rats not receiving ergosterol. The ash of the group receiving neither irradiation nor ergosterol was significantly lower than that of the other groups. The group which received ergosterol but not irradiation showed an ash content of the bones which did not differ significantly from that of the two irradiated groups.

3. No answer therefore was obtained to the main question of the enquiry, *i.e.* the possibility of ergosterol synthesis in the rat body. Since however, when the supply of ultra-violet light was abundant, a variation in the amount of ergosterol in the diet made no difference to the calcification, it must be concluded that the rat was, in those circumstances, sufficiently supplied with ergosterol. Such ergosterol may have been supplied by some measure of

synthesis or it may have been derived from a residual amount in the diet or from the faeces, for in spite of all precautions it was found impossible to prevent the rat from consuming traces of the latter.

4. When, however, the supply of ultra-violet light was very small, the supply of ergosterol from any of the possible sources just mentioned was not sufficient to promote the maximum calcification, which was only then attained when abundant ergosterol was added to the diet.

5. In the course of these experiments, which represent a prolonged maintenance of rats on a fat-free diet, the animals developed the "scaly tail" condition of Burr and Burr, who attributed it to lack of fat in the diet. The observation is discussed in the next paper.

The writers are indebted to the Medical Research Council for a whole time and a half-time grant respectively, and also wish to express their thanks to Sir Charles Martin for help and criticism, to Mrs Smedley-MacLean and to Dr Rosenheim for help and advice in preparing the concentrate of vitamin A and to Dr Harriette Chick for similar assistance in preparing the concentrate of the B vitamins.

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XXXVII. THE RELATION OF A FAT-FREE DIET TO THE SCALY TAIL CONDITION IN RATS DESCRIBED BY BURR AND BURR.

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Experiments made before the discovery of the fat-soluble vitamins with the object of determining the indispensability or otherwise of fat in the diet have little value. No effort was made to supply the fat-soluble vitamins and failures which were really due to a deficiency of them were attributed to the lack of fat as fat.

As early as 1918 Hindhede [1918] concluded from prolonged dietary experiments on young men that fat was not necessary if a daily ration of fruit and vegetables, sufficient to supply the fat-soluble vitamins, was consumed.

Animal experiments in which the distinction was drawn between fat as fat and fat as the vehicle for the fat-soluble vitamins were first made by Osborne and Mendel [1920–21] and by Drummond and Coward [1921]. In the observations of Osborne and Mendel the diets were not completely devoid of fat, they were only relatively very low and fat-soluble vitamins were supplied as dried alfalfa. The rats throve and grew well and the authors concluded that "if true fats are essential for nutrition during growth the minimum necessary must be exceedingly small." In Drummond and Coward's experiment the fat in the diet was reduced to a trace and fat-soluble vitamins were supplied as the unsaponifiable fraction of fish-liver oil. The rats grew well on the diet and reproduced, and the conclusion of Hindhede and of Osborne and Mendel was completely borne out, that fats, while being a convenient form of energy-bearing food, are only indispensable as carriers of the fat-soluble vitamins; when a supply of these is otherwise assured, fats can be omitted from the dietary.

For some years this view remained unchallenged, until Evans and Burr [1928] and Burr and Burr [1929] announced a new dietary deficiency in fat-free diets, which was relieved by the administration of fatty acids. The rats on the fat-deficient diet grew sub-normally and developed a scaliness and annulation of the tail, which might become necrosed, so that 1–3 cm. of the tip might be lost; the skin of the feet and also of the whole body ultimately became affected and the animals finally declined and died.

McAmis, Anderson and Mendel [1929] describe some experiments in which

rats on fat-free diets grew and throve less well than those on diets containing fat, though the fat-soluble vitamins were supplied as the unsaponifiable fraction of cod-liver oil. One case is described where the tail of the rat became dark and gangrenous, but a condition as severe as that described by Burr and Burr was not observed.

The condition of "scaly tail" which occurred among the rats used by the present writers in the experiment described in the preceding paper [1931] appears to be identical with that described by Burr and Burr, but as has already been stated, it could not be referred to the lack of fat in the dietary, since it occurred also among rats receiving abundant fat. The factors to which it might be attributed appeared to be rather the type of caseinogen, the source of B vitamins and the type of cage used, with open wire grids to prevent coprophagy. The condition has also been observed in the Lister Institute by Chick, by Roscoe and by Aykroyd (personal communications) on a variety of diets but always when using cages with coarse wire flooring, on diets containing specially purified caseinogen and usually with yeast derivatives and yeast fractions, rather than with whole yeast as the source of B vitamins. Experience in this Institute is not incompatible with the view that modification of the supply and source of yeast vitamins, under which heading the use of anti-coprophagy cages is included, is the responsible factor in the production of scaly tail.

Analysis of the experimental results of the other workers already cited is not inconsistent with such an interpretation. The early experiments of Drummond and Coward and of Osborne and Mendel, in which the rats throve on a fat-free diet, would probably have been carried out in cages with solid bottoms as was usual at that date. Evans and Burr made a first series of experiments in which cages with solid bottoms were used, while in all the subsequent ones the rats were housed on wire grids. It is remarkable that while in each experiment rats receiving fat grew better than those which did not, yet those which received fat and were caged on wire grids grew less well than ones which did not receive fat, but were housed in cages with solid bottoms. Less pure caseinogen seems also to have been used in the experiment where the cages had solid bottoms. The B vitamins were supplied as 0·7 g. daily of Fleischman yeast. No symptoms are described as having occurred in this experiment.

In the experiment of McAmis, Anderson and Mendel the conditions were somewhat similar; the experiments were carried out using, partly, cages "consisting largely of glass" and, partly, cages with floors of open wire mesh. Harris yeast concentrate was used as a source of the B vitamins and later it was supplemented with 400 mg. of yeast daily. Inferior growth was shown by the rats on the fat-free diet and in one instance a tail lesion was observed, but the experiments are not well described and it is clear from the account of the autopsies that the rats on the fat-free diet, which received their fat-soluble vitamins as unsaponifiable fraction of cod-liver oil, were partly de-

ficient in vitamin A. It is not possible to obtain much evidence from an analysis of these experiments.

In the work of Burr and Burr the rats were housed throughout on coarse wire grids (two meshes to the inch); they received a diet which contained no fat, specially purified caseinogen and ether-extracted yeast. In these conditions the symptoms already described developed severely and could be cured by 10 drops of lard or 1·0 g. of liver daily, but not by 0·7 g. of whole yeast daily. The conditions here were more drastic and the syndrome developed fully, but the cure with 10 drops of lard and the failure with 0·7 g. whole yeast do not appear to support a theory of some deficiency in the yeast vitamin complex. It might however be that in the drastic conditions of the experiment 0·7 g. of dried yeast did not supply enough of the factor, after the condition had developed severely. Aykroyd (personal communication) also only secured slow improvement of the condition when established to a severe degree, with 1·0 g. of dried yeast but a cure was brought about with a daily dose of 1·0 g. dried liver. The failure with 0·7 g. of dried yeast definitely puts any question of a deficiency of vitamin B_2 out of court.

Some work of Evans and Lepkovsky [1929] however suggests in a most interesting manner that the curative action of fat for scaly tail may be exercised by making good some deficiency in the yeast vitamin complex. They show that fat in the diet can have the effect of reducing the amount of B vitamin which the rat needs and conclude that the more fat there is in the diet up to a point the less antineuritic vitamin B_1 is needed. They do not consider that this sparing action of fat is extended to vitamin B_2, but a careful examination of their charts suggests that it is, though less effectively than in the case of vitamin B_1. If fat, therefore, can have a sparing action on the B vitamin complex, an explanation is offered of Burr and Burr's observations, which is also compatible with an ascription of the deficiency which produces scaly tail to lack of some factor within the yeast vitamin complex. Evans and Lepkovsky go no further than conjecture with regard to the mechanism by which the sparing action is produced; they suggest possible alterations in the rat's intestinal flora. The present writers would tentatively suggest, though they have no evidence in support of their suggestion, that the addition of certain fats might operate by altering the consistency of the faeces, rendering them more pasty and less liable to fall through the wire grids and thereby promoting coprophagy. At any rate an examination of the literature and a comparison of the results with the writers' own experience made it seem worth while to test further the hypothesis that the aetiology of the scaly tail syndrome lies in variation of the conditions governing the supply of the yeast vitamin complex.

Experimental.

The object aimed at was to compare the condition of rats which received diets containing fat or no fat, and were kept on wire screens and on solid bottoms respectively.

It was decided to use the commercial yeast extract marmite as the source of B vitamins.

Twelve rats were used which were derived from four different litters, so that it was not possible to balance four experimental groups perfectly in this respect.

For the first 50–60 days of experiment all the rats received the following diet:

Caseinogen (British Drug Houses, fat- and vitamin-free) ...	20
Rice starch	72
Marmite yeast extract	4
Salt mixture (McCollum 185)	4

Vitamin D was given separately as 0·004 mg. of irradiated ergosterol in ethyl oleate daily, but no vitamin A was given in this period of the experiment as the animals were originally intended for another purpose.

The rats were kept in cages with a floor of such finely perforated zinc that, so far as coprophagy was concerned, it amounted to a solid floor.

About the 50th–60th day the animals showed signs of failing from lack of vitamin B_1; marmite was therefore increased to 15 % of the diet. At the same time fat was introduced into the diet of six rats as 10 % of hardened arachis oil in the place of 10 % of starch, while the remaining six rats remained on the same diet devoid of fat. Of the six rats receiving fat in the diet, three were placed in cages with coarse screens (9 to the square inch) and three remained on the same floors as before: the six on a diet without fat were similarly divided. The irradiated ergosterol from this point also was administered in solution in liquid paraffin instead of in ethyl oleate. Experiments with carotene had shown how quickly it is oxidised in ethyl oleate solution, and it was feared that vitamin D might suffer oxidation in the same way.

For the next 40 days, for the purpose of another observation, the six rats on fine screens received their vitamin A as 0·006 mg. of carotene in liquid paraffin daily, while those on coarse wires received it as 0·04 g. of fresh green cabbage daily. At the end of the 40 days, *i.e.* about the 100th day of experiment, all the rats received their vitamin A as cabbage. At the same time, since any symptoms of scaly tail so far shown were very slight, the marmite was reduced once more to 8 %; it was deemed that the supply of vitamins B_1 and B_2 would be again endangered if it were reduced any lower.

About the 180th day as the rats on the fine wires showed very little sign of the disease coarse screens were substituted.

About the 200th day, when it was clear that fat in the diet was making no difference, vitamins A and D were administered more conveniently as 5 drops of cod-liver oil daily, instead of as cabbage and irradiated ergosterol.

The total duration of the experiment was about 290 days.

On the regime above described, no rat developed a severe condition of scaly tail but all showed a recognisable degree of it, except one which died

on the 126th day. At autopsy it was found to have a cystitis, with stones in the bladder; the vitamin A supply was therefore probably insufficient for it. The growth of the animals is not considered in the results; the supply of vitamins B_1 and B_2 was insufficient for good growth over the major part of the experiment, so that the growth performances have no significance. The behaviour of the groups was as follows.

Group 1. Diet + fat. Coarse screens. Symptoms developed early and comparatively severely, with a well-marked black necrotic tip to the tail in one case, moderately in the second case and slightly in the third. The most severe case showed slow improvement when treated with 0·2 g. dried yeast for 72 days and became cured, so that even the black tip to the tail disappeared, when treated with 0·4 g. daily for 41 days. The second case improved very slowly on 0·1 and 0·2 g. daily of dried yeast, but was almost cured when the experiment was ended after it had been for 21 days on 0·4 g. The third showed little improvement when treated with 0·2 and 0·4 g. of autoclaved (120° for 5 hours) dried yeast daily but was improved after 27 days on 0·4 g. of dried unheated yeast.

Group 2. Diet − fat. Coarse screens. All the three rats developed well-marked scaliness and two showed very small necrotic black tips to their tails, one of which made its appearance while the rat was under treatment with autoclaved yeast. Treatment with 0·4 or 0·5 g. of autoclaved yeast brought about no improvement, but when the dose was changed to one of 0·4 g. of yeast, not autoclaved, over a period of 20 days, two of the rats showed improvement and one was cured.

Group 3. Diet + fat. Fine screens. Symptoms developed very slightly while the rats were on the fine screens but increased after coarse ones were substituted. One rat died without symptoms before that date was reached. The two survivors were treated with 0·2 and 0·5 g. autoclaved yeast with no benefit; a black tip to the tail was developed in one case after the treatment had begun. Improvement took place when the rats were treated with 0·4 g. dried yeast for 12 and 27 days respectively.

Group 4. Diet − fat. Fine screens. The tail of one rat remained normal up to the removal of the fine screens; the other two showed slight but slowly increasing symptoms. After the substitution of coarse screens the degree of scaliness increased and two rats developed black tips to their tails, one of them whilst under treatment with autoclaved yeast. No improvement took place with doses of 0·2, 0·4 or 0·5 g. of autoclaved yeast. With a daily dose of 0·4 g. of dried yeast improvement took place after 10, 17 and 18 days respectively.

Discussion.

The condition was not developed severely enough to give very sharp results. On 15 % of marmite in the diet, it scarcely developed at all; it was only after the marmite was reduced again to 8 % that the condition became definite. Symptoms occurred approximately equally in the groups with and without

fat, though they were perhaps slightly more severe in the groups without fat. Four of the six rats without fat developed black tips to their tails, while only two did so in the two groups which received fat. On the other hand the most severe case, with the largest black necrotic area on the tail, was that of a rat which received fat in the diet.

The symptoms developed slightly or not at all when the rats were housed on fine screens and became more marked when coarse ones were substituted.

The condition was unaffected or even grew worse while the animals were being treated with 0·2–0·5 g. daily of autoclaved yeast. Dried yeast in a daily dose of 0·4 g. produced improvement, and the most severe case, on which treatment was begun early, was completely cured.

The caseinogen and the coarse wire grids were the same as were used in the sterol experiment, where the symptoms were more marked; the source of yeast vitamins was however different and one is inclined to suggest that in marmite the vitamin factors are not sufficiently unbalanced for the production of scaly tail. Even 0·4 g. of yeast only slowly cured the condition when it was very mild; it is easy to imagine that considerably larger amounts might fail to cure a very much more drastic deficiency.

The results of this experiment lend some support to the hypothesis that the production of the scaly tail condition is influenced by factors which influence the supply of the yeast vitamin complex. In certain circumstances the presence or absence of fat in the diet appears to be one of these factors, but in the conditions of the present experiment and using hardened arachis oil as the form of fat, it was not appreciably operative. Burr and Burr used lard, and Evans and Burr lard, butter-fat, corn oil and coconut oil but no hardened fat, but Evans and Lepkovsky found that the sparing action of fat was also exercised by hardened cottonseed oil, so that it seems improbable that the virtue is lost in the process of hardening. The failure of fat to exercise its protective action against the development of scaly tail in the writers' experiments therefore remains unexplained, but it seems to exclude the hypothesis that scaly tail is the direct result of a pure fat deficiency.

Summary.

1. A scaly tail condition, considered to be the same as that described by Burr and Burr and ascribed by them to lack of fat in the diet, was developed in the course of experiments in which the rats received diets with and without fat.

2. The condition was developed when the rats were kept on coarse wire grids, with highly purified caseinogen and usually when extracts of yeast, rather than whole yeast, were used as the source of B vitamins.

3. A comparison of the behaviour of rats housed on coarse and fine grids showed that the development of the condition was favoured by the use of the former.

4. The condition, when developed in a mild degree, could be cured by

the addition of whole dried yeast. Autoclaved yeast conferred no benefit, showing that the condition could not be due to an insufficiency of vitamin B_2.

5. The literature is discussed and it is suggested that the scaly tail condition may be correlated with some deficiency in the yeast vitamin complex and is influenced by the same factors as influence the action of the B vitamins, *i.e.* opportunities for coprophagy and the amount of fat in the diet.

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XXXVIII. THE MICRO-DETERMINATION OF BARIUM.

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(*Received January 8th, 1931.*)

In the estimation of the barium present in barium hexosephosphates the ordinary macro-method on a small scale has been employed in this laboratory in the past. Sufficient of the salt to give about 20 mg. $BaSO_4$ was used, the barium precipitated in the boiling solution by the addition of very dilute sulphuric acid, and the barium sulphate filtered, ignited in the usual way and weighed on a micro-balance. The results obtained were often somewhat low and the agreement among duplicates was not always as good as was desirable. An investigation of the probable sources of error was made.

The error due to the use of coal gas for heating the barium sulphate.

It was found that heating the small platinum crucible to redness by means of a Bunsen burner to drive off the excess sulphuric acid after treatment of the incinerated residue with hydrochloric and sulphuric acids caused a considerable and variable loss in weight, due to reduction of some of the barium sulphate to barium sulphide in the presence of the hot, reducing gases from the burner. This loss can be eliminated by heating electrically at this stage, as shown by Table I.

Table I.

	Weight of barium sulphate (mg.)	Manner of heating
	17·200	Gas
	21·300	Gentle gas heating, no blasting
	20·205	" "
	17·625	Gas. Blasting
	16·850	"
	18·275	"
	19·395	Electrical
	19·346	"
Another sample	18·654	Electrical
	18·646	"
	15·185	Gas
	18·667	Electrical

Between each of the weighings recorded above the barium sulphate was treated with 4 drops of 2*N* HCl, followed by 4 drops of 2*N* H_2SO_4 and the moisture driven off by gently heating the crucible lid prior to heating, in the manner indicated in Table I, to drive off the sulphuric acid. In every case

after gas-heating there was a strong odour of H_2S on adding the hydrochloric acid, whereas after electrical heating there was no odour.

The error due to incomplete filtration.

It was found that loss of barium sulphate due to incomplete filtration could be eliminated by heating the precipitated barium sulphate and supernatant liquid for 6 to 8 hours at incipient boiling, in the usual manner, leaving at 0° overnight, filtering cold and refiltering the filtrate through the same paper (Whatman 44). Previously, it had been found that on filtering hot and without re-filtration the loss from three barium determinations, each of which gave rise to about 18 mg. $BaSO_4$, was 4·162 mg. This loss was determined by evaporating to dryness the combined filtrates from the three determinations and subtracting the weight due to solid impurities in the reagents employed. Barium chloride had been used as the source of barium so this was permissible.

In order to demonstrate the accuracy of the method the following experiment was performed. Approximately 10 mg. of recrystallised barium chloride were dissolved in 100 cc. of water. Six 2 cc. samples were taken with an Ostwald pipette. Three of these were treated according to Pregl's method for the micro-estimation of chlorine. The other three were treated according to the method to be described for the estimation of barium:

$BaSO_4$ obtained, mg.	22·530	22·503	22·530
After re-treating with HCl and H_2SO_4 and re-heating	22·535	22·515	22·530
Average	22·525 mg.		
AgCl obtained, mg.	27·720	27·760	27·715
Average	27·732		
Calculated as $BaSO_4$	22·581 mg.		

The combined filtrates (*ca.* 100 cc.) from the three barium determinations were evaporated to dryness and the residue weighed. The same quantities of the same reagents used in the three barium determinations were evaporated to dryness and the residue weighed. Results:

Residue obtained from the three filtrates	1·268 mg.
Residue obtained from the reagents	1·054 mg.
$BaSO_4$ in the combined filtrates	0·214 mg.
Loss of $BaSO_4$ in each determination ($\frac{1}{3} \times 0{\cdot}214$ mg.)	0·071 mg.

Thus, the weight of barium sulphate found agrees closely with the figure obtained by calculating the equivalent weight in barium sulphate of the silver chloride found, and the loss of barium sulphate is not more than 0·3 % of the total weight. This loss may be even less than that shown since any soluble impurity which may have been present in the recrystallised barium chloride could not be determined, and would, of course, be present in the residue obtained on evaporation of the combined filtrates. Also, agreement is found in the weights of barium sulphate before and after re-treatment with hydrochloric and sulphuric acids and re-heating. It may be noted that even when

evaporated to a small volume the combined filtrates from the three barium determinations showed no sign of precipitate even on standing for 24 hours.

Details of the method employed.

Place sufficient of the material to be analysed to give about 20 mg. $BaSO_4$ in a 150 cc. beaker, add water to 100 cc. and heat to incipient boiling. In a bulb of about 15 cc. capacity, which has been drawn down to a capillary at one end, place 10 cc. of water containing one drop of $2N$ H_2SO_4 and allow this solution to drip slowly into the solution in the beaker. Then add 1 cc. of $2N$ H_2SO_4 in 10 cc. of water in the same manner. Finally, add 1 cc. of $2N$ H_2SO_4 directly to the beaker. Cover the beaker with a clock-glass and maintain it at approximately 90° for 6 to 8 hours, thereby reducing the volume of the solution to about 20 cc. Set aside over night at 0°. Filter through a small filter-paper (4 to 5 cm.), stirring up the precipitate before each addition of liquid to the funnel. Never allow the surface of the liquid in the funnel to rise closer than 1 mm. to the top of the paper, thereby avoiding a ring of barium sulphate on the glass. When the liquid has all been filtered return the filtrate to the original beaker and repeat the filtration. In this way most of the precipitate is transferred to the paper. Transfer the remainder by using small portions of the filtrate. Finally, wash the beaker and precipitate with distilled water. Dry the filter-paper in the funnel in an oven at 100° and transfer it to a small, weighed platinum crucible. Char the paper slowly by heating the projecting lid only of the crucible with a small flame, never allowing the paper to burn. When charred, increase the heat gradually, finishing with strong heating to complete the incineration. Allow the crucible to cool. Add 2 drops of distilled water, making sure that the precipitate and filter ash are wet. (Otherwise, spattering will occur due to vigorous liberation of hydrogen sulphide on the addition of hydrochloric acid.) Add 1 drop of $2N$ HCl. Wait till the usual effervescence has moderated before adding 3 or 4 more drops. Add 4 or 5 drops of $2N$ H_2SO_4. Drive off the moisture by heating the projecting lid only of the crucible with a very small flame, avoiding any approach to boiling. Finally, heat the crucible for 20 to 25 minutes on an electric heater to drive off the sulphuric acid. Placing the crucible as close as possible to a coil of red-hot resistance wire has been found to provide sufficient heat. It is advisable to remove the lid and heat it separately since, unless an electrically heated muffle is used, there is a tendency for the sulphuric acid to condense on the lid. Place the crucible on the copper block as used by Pregl. After 10 minutes transfer it to the micro-balance case and weigh it after a further 15 minutes. The empty crucible should, of course, have been weighed in the same manner. Repeat the operations from the point where the hydrochloric acid was added. The second weight should agree with the first within 0·03 mg.

XXXIX. THE METHYLATION OF HEXOSEMONOPHOSPHORIC ESTER.

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In the original description of hexosemonophosphoric ester prepared by the action of yeast juice on fructose and glucose Robison [1922] expressed the opinion that the compound was probably a mixture of glucose- and fructose-monophosphoric esters. This conclusion was based on the properties of the dextrorotatory sugar obtained by acid hydrolysis of the ester and on the positive Selivanoff reaction for fructose given by all samples of the purified hexosemonophosphoric acid.

Meyerhof and Lohmann [1927] obtained further experimental evidence that the hexosemonophosphoric acid described by Robison was a mixture of an aldose- and a ketose-phosphoric ester. By estimating the reducing power according to Bertrand's method and comparing this figure with that obtained when using the Willstätter and Schudel [1918] hypoiodite method they were able to calculate the percentage of aldosemonophosphoric ester present in the total hexosemonophosphoric acid. Lohmann [1928] attempted to separate these aldose and ketose constituents of hexosemonophosphoric ester by acid hydrolysis, since in the event of the ketosemonophosphoric acid present being identical with the fructosemonophosphoric acid prepared by Neuberg [1918] it should be more easily hydrolysed than the remaining aldosemonophosphoric acid. After partial hydrolysis Lohmann obtained an ester which showed an enhanced aldose content together with a specific rotation $[\alpha]_D^{18^\circ} + 35{\cdot}7^\circ$ for the free acid. He did not succeed however in completely eliminating the ketose component from the aldosemonophosphate.

Robison and King [1929] by fractional crystallisation of the brucine salt of hexosemonophosphoric ester obtained a compound possessing a higher dextrorotation ($[\alpha]_{5461}^{20^\circ} + 21^\circ$ for the barium salt) than the original ester and an increased content of aldosemonophosphate (91 %) as measured by the iodimetric method modified according to Macleod and Robison [1929].

Towards the end of 1928 Robison and King had separated the aldosemonophosphate from yeast hexosemonophosphate and had obtained strong evidence that it was a monophosphoric ester of glucose. Their experiments yielded

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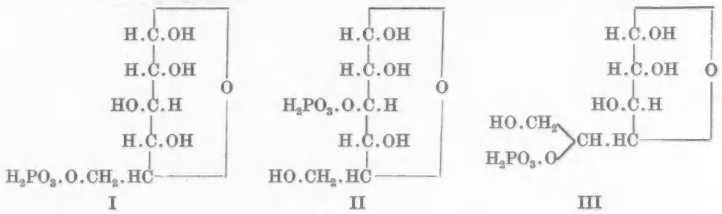
sufficient of this purified aldosemonophosphate to enable work to be commenced on its methylation with a view to proving its structure.

The results of the methylation of this hexosemonophosphate were given in a short abstract by King and Morgan [1929, 1]. It was shown that if this hexosemonophosphate was methylated by Fischer's method, both at room temperature and at 60°, two distinct hexosides were formed. The hexoside produced at room temperature was rapidly hydrolysed by 0·1 *N* acid at 35°, the methyl-group being completely eliminated in about 24 hours. The hexoside produced by methylation at the higher temperature was not hydrolysed under these conditions. These two hexosides probably represent the reactive (furanose) and the stable (pyranose) forms whose formation could only take place if the hydroxyl-groups attached to the carbon atoms 4 and 5 are unsubstituted. It appeared therefore that the phosphate group was not in either of these positions. The earlier work of Robison [1922] had eliminated the possibility of the phosphoric acid group being attached in positions 1 and 2. There remained however positions 3 and 6. King and Morgan pointed out that while no definite conclusions could be reached as to the exact position of the phosphoric acid group, the rate of hydrolysis of this group pointed to position 6 as the most probable (I) but this inference appeared to be incompatible with the difference in the melting points of the osazones of hexosemonophosphoric acid and hexosediphosphoric acid which had been reported by Robison. It was therefore suggested [King and Morgan, 1929, 2] that the phosphoric acid group was in position 3 (II). Levene and Raymond [1929] having observed the formation of a reactive γ-glucoside when hexosemonophosphoric acid was methylated by Fischer's method at room temperature, concluded that this



phosphoric ester possessed an unstable furanose ring, the phosphoric acid group being attached to the fifth carbon atom (III). Methylation at these temperatures is, however, considered to favour the production of a γ-hexoside and, therefore, cannot be used as evidence that the phosphoric acid group is in the fifth position and has thus prevented the formation of the normal 1:5 ring.

Josephson and Proffe [1930] converted di-*iso*propylideneglucose by the action of phosphoryl chloride in pyridine solution at $-18°$ to $-20°$ into a di-*iso*propylideneglucose phosphate which on treatment with baryta and subsequent hydrolysis yielded a glucose-3-phosphate possessing a pyranose ring

structure. These authors claim that Robison's ester is not identical with their synthetic ester and, therefore, cannot be a glucose-3-phosphoric acid as suggested by King and Morgan.

The methylation experiments quoted in the original abstract of King and Morgan have been repeated using further preparations of purified aldosemonophosphate and the production of the two different types of hexosides has been confirmed. The evidence as to the nature of the oxide ring system in these two different types of hexosides has been further developed by comparing the rate of hydrolysis of the dephosphorylated methylhexosides with a methylhexoside of known structure. In the absence of any evidence which points to the migration of the phosphoric acid group during methylation it would appear highly probable that positions 4 and 5 in aldosemonophosphoric acid are not substituted by the phosphoric acid group. Meanwhile Robison and King [1929, 1931] isolated the pure aldosemonophosphoric acid component of hexosemonophosphoric acid and the determination of the melting point of its osazone showed this to be identical with that of the osazone of fructosemonophosphoric acid derived from fructosediphosphoric acid in which the phosphoric acid group is most probably in position 6 [Morgan and Robison, 1928; Morgan, 1929]. From this evidence the aldosemonophosphoric acid would appear to be an aldose-6-phosphoric acid and this conclusion is to some extent supported by the recent work of Levene and Raymond [1930]. These authors have also prepared the synthetic glucose-3-phosphoric acid and have shown that it differs markedly in properties from the Robison hexosemonophosphate. They have also prepared another synthetic ester which they consider to be glucose-6-phosphoric acid and which resembles the Robison ester in some though not in all respects [Robison and King, 1931].

Experimental.

The hexosemonophosphoric ester used in the methylation experiments was prepared by the fractional crystallisation from 20 % ethyl alcohol of the brucine salt of the crude ester isolated from the products of yeast juice fermentation, and a typical analysis of the purified barium hexosemonophosphate is given below.

Total phosphorus	7·6 %
Inorganic phosphate	Absent
Reducing power (H. and J.) ...	35·4 %
Iodine reduction (M. and R.)...	39·3 % (≡ 87 % aldose component)
$[\alpha]^{18°}_{5461}$	+ 20·4°

Methylation of the hexosemonophosphate.

Methylation at 18°. Barium hexosemonophosphate (9·2 g.) was added to 200 cc. of anhydrous methyl alcohol containing hydrogen chloride in amount

calculated to give a final solution containing 0·5 % of free hydrogen chloride. To facilitate the rapid solution of the salt the mixture was shaken under conditions previously described in detail by Morgan [1927]. The barium salt dissolved rapidly to give a clear pale yellow solution and no separation of barium chloride occurred during the esterification. The progress of the esterification was followed by estimating the reducing power of the solution by the method of Hagedorn and Jensen and by frequent readings of the rotation in a 4 dm. polarimeter tube. During the first hour there was a sharp rise in the observed rotation followed by a sudden drop, the rotation continuing to decrease until esterification was complete. The course of this change is shown in Fig. 1, Curve A, while the analytical figures obtained during the esterifica-

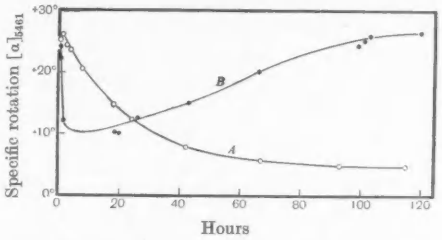


Fig. 1. Change in optical rotation during methylation of hexosemonophosphoric acid.
A. At 18°. B. At 60°.

Table I.

Time		Observed rotation	Total phosphorus	Reducing power as glucose per 1 cc. of the solution	Esterification
Hrs.	Mins.	$[\alpha]_{5461}$	g.	mg.	%
	5	4·67°	0·692	14·8	0
	10	4·74	—	—	—
	25	4·77	—	—	—
	40	4·79	—	—	—
	55	4·81	—	—	—
1	25	4·67	—	—	—
2	10	4·49	—	—	—
3	30	4·36	—	—	—
7	25	3·79	0·691	11·70	21
18		2·72	0·694	9·40	36
24		2·72	—	—	—
42		1·43	0·685	6·45	56
67		1·03	0·682	5·35	64
93		0·88	0·687	3·95	73
115		0·82	0·689	3·05	79
160		0·79	0·690	2·70	85
211		0·63	—	1·32	91
*260		—	—	0·12	99

* After re-solution in fresh methyl alcoholic hydrogen chloride.

tion are given in Table I. There was no formation of inorganic phosphate during the experiment. The reduction at the end of 8 days indicated that 90 % of the theoretical quantity of hexoside had been produced. The yield of methylhexosidemonophosphate was, however, considerably increased by

neutralising the 90 % equilibrium mixture, distilling off the methyl alcohol under reduced pressure at 35°–40°, and redissolving the hexose-hexoside mixture obtained in 200 cc. of dry methyl alcohol containing hydrogen chloride as described above. Under these conditions the solution became almost non-reducing (less than 1 %) and the methylhexosidemonophosphoric ester was obtained in an almost quantitative yield. The acid methyl alcohol solution of the hexoside was neutralised by the addition of silver carbonate, the precipitated silver chloride filtered off and the filtrate made just pink to phenolphthalein by the addition of methyl alcoholic solution of baryta. The precipitated barium methylhexosidemonophosphate was washed with anhydrous methyl alcohol and dried in a vacuum desiccator. The crude barium salt thus obtained was dissolved in 10 parts of water and fractionally precipitated with increasing concentrations of ethyl alcohol. The following fractions were collected:

Alcohol concentration	Weight of fraction
0–30 % (by volume)	3·3 g.
30–40 % "	2·1 g.
45–80 % "	4·5 g.

The optical rotation of these fractions showed no appreciable difference, thus indicating no separation into the α- and β-methylhexosidemonophosphates. That the α- and β-isomers were present seemed certain from the type of the methylation curve (Fig. 1, Curve A), the preliminary rise being due to the formation of a hexoside possessing greater dextrorotation than the original mixture of α- and β-hexosemonophosphoric acids. In the same way the subsequent fall in rotation presumably indicated the formation of the more laevorotatory isomer.

In another methylation under similar conditions 6·1 g. of barium hexosemonophosphate were dissolved in 300 cc. of anhydrous methyl alcohol containing hydrogen chloride. The reaction was stopped by the addition of excess barium carbonate when 93 % esterification had been reached. The solution was filtered and a hot saturated aqueous solution of baryta added to the filtrate until it was just pink to phenolphthalein. Two volumes of light petroleum and half a volume of ether were added, the resulting precipitate was collected by centrifuging and dried in a vacuum desiccator. The crude barium salt thus obtained, containing barium chloride as the principal impurity, was dissolved in 8 times its weight of water. The barium chloride was precipitated by the addition of a saturated aqueous solution of silver sulphate, the end-point being determined by centrifuging a small sample of the liquid and testing with silver sulphate solution. A slight trace of barium chloride amounting to, at most, 0·15 % of the total solid was left in the solution. The resulting precipitate was centrifuged off and washed with water. The large volume of centrifugate was reduced *in vacuo* over concentrated sulphuric acid to approximately the original volume, when a dark-coloured solution was obtained. To this solution slightly more than sufficient normal sulphuric acid

to give the free hexoside was added. The barium sulphate was quickly centrifuged off, the remaining trace of silver chloride being also removed. The clear colourless solution was then made pink to phenolphthalein by the addition of a cold saturated solution of baryta. The solution was then evaporated to the original volume and centrifuged to remove barium sulphate resulting from the slight excess of sulphuric acid. It was then treated with a mixture of alcohol, ether and light petroleum. The resulting precipitate, after drying over sulphuric acid *in vacuo*, gave the following analytical figures:

	% OCH_3	% P	% Ba
Found	7·57	7·45	32·98
Calculated for $C_6H_{10}O_4(OCH_3)(PO_4Ba)$	7·57	7·57	33·55

Methylation at 60°. Barium hexosemonophosphate (6·0 g.) was added to 100 cc. of anhydrous methyl alcohol containing hydrogen chloride. The general method and precautions observed were the same as those described above. A portion of the methyl alcoholic solution was at once transferred to a jacketed 4 dm. polarimeter tube which was kept, together with the remaining part of the solution, at 60° in an electric oven. At the end of the first hour barium chloride began to crystallise from the alcoholic solution, thus making it necessary to filter the solution before the polarimeter readings could be taken. The course of the methylation was followed and the resulting methylhexosidemonophosphate was isolated as described for the first methylation at 18°. The figures obtained are given in Table II. No inorganic phosphate was

Table II.

Time		Observed rotation	Total phosphorus	Reducing power as glucose per 1 cc. of the solution	Esterification
Hrs.	Mins.	$[\alpha]_{5461}$	g.	mg.	%
	12	5·22°	—	15·2	—
	40	4·79	0·430	10·8	29
1	20	2·62	—	—	—
18	30	2·20	—	5·0	67
20		2·15	—	—	—
26		2·71	—	—	—
41		3·25	0·410	2·3	85
66		4·35	—	1·1	93
99		5·25	—	—	—
101		5·45	—	—	—
103		5·62	0·408	0·6	96
120		5·75	0·410	—	—

liberated during the methylation. It will be seen that the curve showing change in rotation during methylation (Fig. 1, Curve B), is entirely different in form from the corresponding curve for the methylation at 18°. The curve shows a rapid fall during the first 20 hours, followed by a steady rise until the esterification is complete, the final value for the observed rotation being higher than the original value for the hexosemonophosphoric acid. In a second methylation at 60° 6·1 g. of barium hexosemonophosphate were dissolved in 300 cc. of anhydrous methyl alcohol containing hydrogen chloride. The separation of the hexoside and its purification by means of silver sulphate were carried out

in the manner already described under the methylation at 18°. On analysis the methoxyl values were found to be higher than that calculated for barium methylhexosidemonophosphate. It was thought that this might possibly be due to partial methylation of the phosphate group, although potassium dihydrogen phosphate under the same conditions showed no evidence of esterification, a result in agreement with that obtained by Morgan [1927]. The brucine salt was prepared and was repeatedly recrystallised from aqueous alcohol and thereafter reconverted into the barium salt. The methoxyl content of the latter, though somewhat lower than before, was still higher than the calculated figure. Under the conditions employed it is possible that the dimethylacetal derivative would also be formed and might account for the high methoxyl value obtained. The regenerated barium salt gave the following analytical results:

	% OCH_3	% P	% Ba
Found	8·23	7·49	32·76
Calculated for $C_6H_{10}O_4(OCH_3)(PO_4Ba)$	7·57	7·57	33·55

Acid hydrolysis of the methylhexosidemonophosphates.

Hexosidemonophosphate formed at 18°. The barium methylhexosidemonophosphate (47·4 mg.) was dissolved in 10 cc. of 0·1 *N* HCl for the determination of the rate of elimination of the methyl group at 37°. The extent of hydrolysis was measured from time to time by estimating the reducing power of the solution by the Hagedorn and Jensen method. The results shown in Fig. 2,

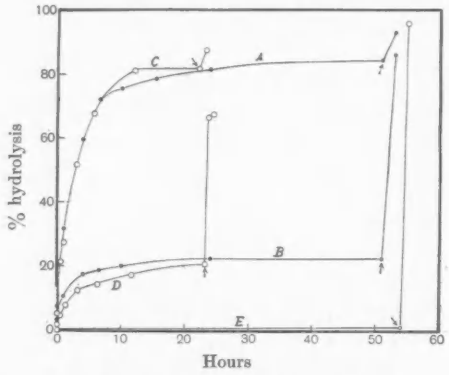


Fig. 2. Hydrolysis in 0·1 *N* HCl at 37°.

A. Methylhexosidemonophosphate from methylation at 18°.
B. Methylhexosidemonophosphate from methylation at 60°.
C. Dephosphorylated methylhexosidemonophosphate from methylation at 18°.
D. Dephosphorylated methylhexosidemonophosphate from methylation at 60°.
E. α-Methylglucoside.
→ At these points the solutions were made *N* with HCl and heated at 100° for the times indicated.

Curve A, indicate that hydrolysis was almost complete at the end of 24 hours. This was confirmed by raising the concentration of HCl to *N* and heating at

100°, when the reducing power, expressed as % of the barium methylhexoside originally present, was increased only from 37·2 to 40·9.

Hexosidemonophosphate formed at 60°. The barium methylhexosidemonophosphate (51·1 mg.) was dissolved in 10 cc. of 0·1 *N* HCl and kept at 37°, the rate of removal of the methyl group being measured as in the previous acid hydrolysis. Curve B, Fig. 2 shows that a small but rapid rise in the reducing power occurred during the first few hours; this was possibly due to the presence of the dimethylacetal derivative mentioned above together with any of the more reactive hexosides which may have been produced during the methylation. No further increase was observed after 24 hours and the reducing power at this point indicated that only 22 % of the total hexoside had been hydrolysed. This amount presumably includes the dimethylacetal derivative, which by calculation from the methoxyl content may have been present to the extent of 20 %. At the end of 50 hours the methylhexoside solution was made *N* with hydrochloric acid and heated at 100°, and after this treatment the reducing power was found to be very nearly equal to that calculated assuming complete hydrolysis of the hexoside.

These experiments indicate that the hexoside produced by methylation at 60° is not hydrolysed to any appreciable extent under conditions which bring about almost complete hydrolysis of the methylhexosidemonophosphate produced at 18°.

The enzymic dephosphorylation of the methylhexosidemonophosphates.

Methylhexosidemonophosphate formed at 18°. A sample of the purified barium methylhexosidemonophosphate (0·503 g.) was dissolved in water and the equivalent amount of potassium sulphate added. The barium sulphate was removed by centrifuging and the solution of the potassium salt adjusted to p_H 8·4 with a dilute solution of potassium hydroxide. The bone phosphatase preparation, 100 mg. [Martland and Robison, 1929], was then added together with a few drops of chloroform, the p_H again adjusted to 8·4 and the enzymic hydrolysis allowed to proceed at room temperature. Estimations of inorganic phosphate showed that 91 % of the phosphorus was eliminated at the end of 72 hours and that no further hydrolysis occurred in a further 24 hours. At this stage the solution was treated with four times its volume of alcohol, and after filtration the alcoholic solution was evaporated to dryness. The residue was extracted with boiling ethyl alcohol and the alcoholic extract filtered and evaporated. The resulting syrup was then extracted with a small quantity of water, filtered and again evaporated to dryness, when it yielded 210 mg. of a syrup which was free from phosphorus and possessed a reducing power of only 4·5 %. After drying over phosphorus pentoxide *in vacuo* the methoxyl was determined:

Found	14·67 %
Calculated for $C_6H_{11}O_5(OCH_3)$	15·98 %

Methylhexosidemonophosphate formed at 60°. The purified barium methylhexosidemonophosphate (0·638 g.) was converted to the potassium salt and treated with 500 mg.[1] of the bone phosphatase preparation under conditions similar to those described above. At the end of 24 hours 93·5 % of the phosphorus was eliminated and no further hydrolysis took place during the next 24 hours. The dephosphorylated hexoside was then isolated and purified as described above. Analysis for methoxyl gave the following results:

Found	12·73 %
Calculated for $C_6H_{11}O_5(OCH_3)$	15·98 %

The methoxyl value indicates that the product contained a considerable proportion of contaminating material which possibly may have been derived from the phosphatase preparation of which an unnecessarily large amount was used for the hydrolysis.

The acid hydrolysis of the dephosphorylated methylhexosidemonophosphates.

A carefully dried sample of the dephosphorylated methylhexosidemonophosphate (0·114 g.) produced by methylation at 18° was dissolved in 40 cc. of 0·1 *N* HCl and kept at 37°. The extent of the removal of the methyl group was measured by estimating the reducing power of the solution by Hagedorn and Jensen's method as in the acid hydrolysis of the methylhexosidemonophosphate. The optical rotation of the solution in a 4 dm. tube was observed during the hydrolysis. The results are shown in Fig. 2, Curve C; Fig. 3, Curve A;

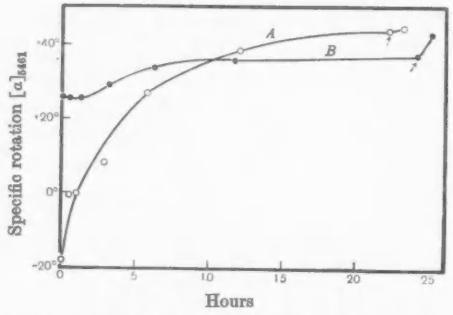


Fig. 3. Change in optical rotation during hydrolysis in 0·1 *N* at 37°.
A. Dephosphorylated methylhexosidemonophosphate from methylation at 18°.
B. Dephosphorylated methylhexosidemonophosphate from methylation at 60°.
→ At these points the solutions were made *N* with HCl and heated at 100° for 1 hour.

and in Table III, and indicate the extreme ease with which the methyl group is eliminated under these conditions. About 80 % hydrolysis was effected in 12 hours and this figure was not increased in the following 12 hours. Heating at 100° for 1 hour in *N* HCl increased the hydrolysis to 87 %. At the end

[1] This amount was used in error, in place of 100 mg.

Table III.

Time		% hydrolysis (H. and J.)	Observed rotation $[\alpha]_{5461}$
Hrs.	Mins.		
0	0	4·87	−0·21°
*0	30	21·29	−0·06
1		27·30	−0·04
2	52	51·70	+0·09
5	44	67·77	+0·30
12		81·32	+0·43
22	15	81·81	+0·47
†23	15	87·72	+0·36

* 39 cc. of the original solution + 0·4 cc. 10 *N* HCl + 0·6 cc. H_2O.
† After heating 30 cc. of the above solution + 8 cc. 10 *N* HCl + 2 cc. H_2O at 100° for 1 hour.

of the hydrolysis at 37° the specific rotation, based on the weight of material taken was $[\alpha]_{5461} + 44·0°$, and on the glucose present as indicated by Hagedorn and Jensen's method was $[\alpha]_{5461} + 55·6°$. After hydrolysis at 37° the reduction given by the iodimetric method of Macleod and Robison was 77·7 %.

Table IV.

Time		% hydrolysis (H. and J.)	Observed rotation $[\alpha]_{5461}$
Hrs.	Mins.		
0	0	1·8	+0·30°
*0	30	4·5	+0·27
1	15	7·7	+0·27
3	10	12·4	+0·32
6	15	14·2	+0·36
11	40	17·1	+0·38
23		20·5	+0·395
†23	30	66·5	+0·40
‡24		67·6	+0·36

* 36·5 cc. of the original solution + 0·4 cc. 10 *N* HCl + 3·1 cc. H_2O.
† After heating 35·2 cc. of the above solution + 4 cc. 10 *N* HCl + 0·8 cc. H_2O at 100° for 30 minutes.
‡ 30 minutes' further heating at 100°.

A sample of the dephosphorylated methylhexoside (0·116 g.) obtained from the product of methylation at 60° was dissolved in water, made up to 40 cc. and the reducing power and rotation of the solution were determined. The course of the hydrolysis was followed by frequent estimations of the reducing power (Hagedorn and Jensen) of the solution and by the change in optical rotation as observed in a 4 dm. tube. Fig. 2, Curve D shows that at the commencement of the hydrolysis there was a rapid but limited rise in the reducing power of the solution indicating the presence of about 20 % of a substance readily hydrolysed by 0·1 *N* HCl at 37°. After 20 hours, as no further hydrolysis appeared to take place, the solution was made *N* with hydrochloric acid and heated at 100° for half an hour. This procedure raised the percentage hydrolysis from 20·5 to 66·5, while after a further 30 minutes at 100° this figure was only increased to 67·6. The iodine reduction value determined at this stage corresponded with 74·9 % hydrolysis. The specific rotation after 30 minutes' heating at 100° calculated on the basis of the glucose present (Hagedorn and Jensen) was $[\alpha]^{20°}_{5461} + 69·6°$ and after a further period of

30 minutes at 100°, + 61·5°. The change in optical rotation of the solution during hydrolysis is shown in Fig. 3, Curve B.

The dephosphorylated methylhexosides derived by enzymic hydrolysis from the products of methylation of aldosemonophosphate at 18° and 60° respectively exhibit the same pronounced difference in their rates of hydrolysis by 0·1 *N* HCl at 37° as do the methylhexosidemonophosphates themselves. It is clear from the curves that the presence of the phosphate group does not materially affect the rate of hydrolysis of the methoxyl group.

In order to compare the rates of hydrolysis of these hexosides with that of a compound of known constitution a sample of recrystallised α-methylglucoside was hydrolysed by 0·1 *N* HCl at 37° followed by *N* HCl at 100°. The results are shown in Fig. 2, Curve E. The reducing power and the specific rotation of the hydrolysis product were determined in a separate experiment in which 0·1327 g. of α-methylglucoside in 50 cc. of 2 *N* HCl was heated at 100° for 1 hour. Calculated from the reducing power determined by the Hagedorn and Jensen method, the hydrolysis amounted to 96·5 %, and the specific rotation $[\alpha]^{20°}_{5461}$ of the product was + 65·9°. The reducing power by the iodimetric method, however, indicated complete hydrolysis and on this basis the specific rotation was + 63·4°. These values for the specific rotation are somewhat higher than that of glucose in aqueous solution ($[\alpha]^{20°}_{5461}$ + 62·5°), but a determination of the specific rotation of glucose in similar low concentration in 2 *N* HCl gave the value + 63·6°, while after heating for half an hour at 100° the reducing power by the Hagedorn and Jensen method showed that some destruction of glucose had taken place (3 %) and the specific rotation, calculated from this reducing power, was + 66·3°.

Separation of the α- and β-methylhexosidemonophosphates.

Small amounts of the barium methylhexosidemonophosphates produced by methylation at 18° and 60° respectively were converted into the brucine salts. The barium was removed by shaking an aqueous solution of these salts with a slight excess of brucine sulphate until the reaction was complete. By allowing the alcoholic solutions of the brucine salts to evaporate slowly several crops of crystals were removed. From the brucine salt of the methylhexosidemonophosphoric ester methylated at 18° crops of crystals yielded, on converting to the barium salt, non-reducing compounds having extreme specific rotations of $[\alpha]^{20°}_{5461}$ + 13° and + 49°, while the brucine salts of the more stable hexoside yielded non-reducing barium salts possessing specific rotations of $[\alpha]^{20°}_{5461}$ + 3·5° and + 39°. These figures indicate a separation into the α- and β-isomers of the corresponding reactive and stable hexosides, but since the brucine salts were not exhaustively fractionated using different solvents it is not possible to claim that the specific rotations are those of the pure isomers.

Discussion.

The simplest and most probable explanation of the experimental results described in this paper is that during the methylation of purified aldosemonophosphate two types of aldosides are formed presumably containing a reactive (furanose) or a stable (pyranose) ring structure according to the temperature of methylation. This explanation assumes that only furanose and pyranose ring structures are formed; this assumption is, however, strongly supported by a large mass of experimental work in the sugar group. At present there is no authentic case of a propylene oxide (1:3) sugar known. The structure of di-*iso*propylidenexylose has been investigated by Haworth and Porter [1928] who originally considered that this compound presented a unique opportunity to investigate the possible existence of a propylene oxide (1:3) ring system. They concluded, however, after careful examination, that this compound possesses a furanose (1:4) ring and thus falls into line with one of the two known oxide ring systems. It would appear, therefore, that in the absence of any evidence indicating that the two hexosides produced from the purified aldosemonophosphate by simple methylation at 18° and 60° are different from the known furanose and pyranose types, we may conclude that the phosphoric acid group is not attached at the 4th or 5th position in the aldosemonophosphate.

It may be pointed out that although the ester used in these experiments contained only 87 % of the pure aldosemonophosphate, since isolated by Robison and King [1931], the presence of the second component could not possibly account for the formation of the two methylhexosides in the yields obtained.

The recent work of Robison and King on the melting point of the osazone of the purified aldosemonophosphate and on the oxidation of this ester points to the phosphoric acid group being attached in position 6, while from the synthetic side the evidence of Josephson and Proffe seems to eliminate the possibility of the phosphoric acid being in position 3, a conclusion which is also supported by Levene and Raymond. On the evidence now available it would therefore appear probable that the aldosemonophosphate component of Robison's original hexosemonophosphate is glucose-6-phosphoric acid.

Summary.

1. The methylation of the aldosemonophosphate component of Robison's fermentation hexosemonophosphate by Fischer's method at 18° and at 60° has yielded two distinct forms of methylhexosidemonophosphate.

2. The methylhexosidemonophosphate produced by methylation at 18° is extremely sensitive to acids. With 0·1*N* acid at 36° it is rapidly hydrolysed yielding a reducing hexosemonophosphoric acid.

3. The corresponding hexoside produced by methylation at 60° is stable in the presence of 0·1*N* acid at 37°. Its rate of hydrolysis closely resembles that of *α*-methylglucoside under the conditions described.

4. The bearing of these experimental results on the constitution of the aldosemonophosphate component of Robison's fermentation hexosemonophosphoric ester is discussed.

In conclusion we wish to express our gratitude to Dr R. Robison for his very helpful criticism and advice.

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XL. HEXOSEMONOPHOSPHORIC ESTERS.

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The hexosemonophosphoric ester isolated from the products of the fermentation of glucose and fructose by yeast juice [Harden and Robison, 1914; Robison, 1922] was considered, from its properties and behaviour on hydrolysis, to be a mixture of isomers, probably the monophosphoric esters of glucose and fructose. The differences observed in the specific rotations of specimens prepared from different fermentation experiments suggested that these isomers occurred in varying proportions.

The presence of aldose and ketose derivatives in this ester was confirmed by Meyerhof and Lohmann [1927], who compared its reducing power as estimated by Bertrand's method with the value found by the iodimetric method of Willstätter and Schudel [1918] and, from the ratio of these values, calculated that a sample of the ester contained 17 % of the fructose derivative. A higher percentage of the ketose component can, however, be deduced from their data if the calculation is based on the Willstätter number, *i.e.* the ratio of total hexose to the iodimetric value. While the reducing power of these esters towards Bertrand's copper solution is always lower than that of the unsubstituted hexose, the reducing power of an aldosephosphate towards hypoiodite should be the same as that of the free aldose, a conclusion supported by Sobotka's [1926] experiments with methylated glucoses and by our present results. On this basis Meyerhof and Lohmann's figures indicate the presence of only 66 % and 68 % aldosemonophosphate in two of their preparations and 57 % in a preparation obtained from this laboratory. The last figure is in agreement with our own value (55 %) for the same preparation (see Table I, No. 1). Lohmann [1928] provided further evidence for the dual nature of the ester by investigating the kinetics of its hydrolysis in *N* HCl. Having shown that the rate of hydrolysis falls considerably after a certain period he made use of this method to remove as much as possible of the more easily hydrolysable ketose ester, thereby raising the proportion of aldosemonophosphate from 68 % to 79 % (based on the Willstätter number) and the $[\alpha]_D$ of the free ester from + 28·5° to + 35·7°. Lohmann concluded that either his residual ester must still contain non-aldose components or the aldehyde oxygen must be linked in two different rings, one of which is not split by hypoiodite.

Similar difficulties have been encountered in our own attempts, continued over several years, to separate the components of hexosemonophosphoric

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ester. A knowledge of the constitution of these components was much to be desired but it was first of all essential to isolate them in reasonably pure condition. The methods employed for this purpose have included the fractional crystallisation of the brucine salt, fractional precipitation of normal and basic barium and lead salts and fractional hydrolysis. Two years ago we had obtained a crystalline brucine salt, which, after many recrystallisations, had the appearance of homogeneity. The corresponding barium salt had $[\alpha]_{5461} + 21°$, but the iodimetric reducing power was still 9 % below that required for a pure aldosemonophosphate. Evidence was obtained that this ester was a derivative of *d*-glucose [Robison and King, 1929] and its methylation was commenced in order to determine the position of the phosphoric acid group [King, McLaughlin and Morgan, 1931]. Work on the further purification of the ester was, however, continued and at length yielded a compound whose iodimetric value corresponded exactly with that required for a pure aldosemonophosphate. The preparation and investigation of this ester and of the other components of hexosemonophosphate are here described.

Preparation of the hexosemonophosphoric esters.

The methods now employed for the preparation of the phosphoric esters and for their preliminary separation according to the solubility of their barium salts have been described in another communication [Robison and Morgan, 1930] in which a general account was given of the quantitative examination of the separate fractions. The material for the present investigation of the monophosphoric esters was provided by the soluble-B fractions from the fermentation of glucose or fructose with yeast juice. These fractions were obtained by addition of basic lead acetate to the 10 % alcohol filtrate after removal of the sparingly soluble barium salts, reconversion of the basic lead salt into the barium salt and extraction of the latter with 10 % alcohol. The soluble-B fractions were in some cases converted directly into the brucine salt, but usually a further purification was first carried out by dissolving them in 10 % alcohol and treating the solution with mercuric acetate as previously described [Robison, 1922]. The monophosphate was reprecipitated as the basic lead salt, which was converted back into the barium salt and purified by solution in 10 % alcohol and reprecipitation with alcohol.

Isolation of the aldosemonophosphoric ester.

Crystallisation of the brucine salt. The barium salt was dissolved in 5 times its weight of water and treated with the amount of $5N$ H_2SO_4 required to precipitate the whole of the barium. The barium sulphate was removed by centrifuging and washed once with water and 3 times with 96 % alcohol. The acid solution was treated with a slight excess of brucine (2·2 g. for each cc. $5N$ H_2SO_4), rather less than half of this amount being added in solid form before centrifuging, the remainder, dissolved in hot 50 % alcohol, after the

final washing was completed. Crystallisation of the brucine salt usually commenced immediately, but the solution was allowed to remain at 0° for at least a week before filtration. The salt was recrystallised by dissolving it in 7 or 8 times its weight of 20 % ethyl alcohol at 40° and allowing the filtered solution to remain at 0°. Two types of crystals were obtained, the first fractions consisting of hard, translucent needles, while later fractions, which separated on allowing the mother-liquors to evaporate spontaneously, consisted partly of needles and partly of hexagonal plates. The separate fractions were repeatedly recrystallised from 20 % alcohol and portions of each were then converted into barium salts by dissolving in 20 % alcohol, adding a solution of barium acetate in slight excess and precipitating the barium hexosemonophosphate with absolute alcohol. The salts were purified several times by solution in water and reprecipitation with absolute alcohol. Analyses showed that the barium salts obtained from the needle fractions contained an increased proportion of aldosemonophosphate while those obtained from the hexagonal plates contained less than 50 % of the aldose component. It was noticed that on recrystallisation of the platelets some clusters of needles were usually obtained, and it was concluded that these two types of crystals represented mixed brucine salts of the aldose- and ketose-monophosphates in different proportions. One of the needle fractions obtained after exhaustive fractionation gave a barium salt, which, by iodimetric estimation, contained 91 % of aldose ester, but, in general, recrystallisation from 20 % ethyl alcohol failed to yield so pure a product. A brucine salt recrystallised 4 times from this solvent, the solution being left at 0° for at least a week at each recrystallisation in order to obtain the maximum crop of crystals, contained only 79 % of the aldose component. The next stage in the purification was effected by recrystallisation of this brucine salt from boiling 90 % or 95 % methyl alcohol, from which it slowly separated, at room temperature or at 0°, in large clumps of soft, opaque, silky needles. After two further recrystallisations a portion of the salt was converted to barium salt, the analysis of which showed that the proportion of aldose ester had increased from 79 % to 90 %. The specific rotation was, however, somewhat lower than that of the 91 % salt mentioned above. The final stage in the purification of the aldosemonophosphate was achieved by recrystallisation from boiling absolute methyl alcohol in which the salt is only sparingly soluble. A small amount of sparingly soluble residue was filtered off and a small fraction of hard, minute crystals which separated rapidly on cooling to room temperature was also removed. The clear solution was then left in a closed vessel at 0° for some days, during which almost the whole of the remaining brucine salt crystallised out. These operations were repeated twice but in the last recrystallisation the crystals were removed by filtration after 24 hours. This fraction and the salt remaining in the mother-liquors were converted into the barium salts which were purified as already described and analysed. The iodimetric value of the salt prepared from the crystals was found to be equal to that required for 100 % aldosemonophos-

phate, while the barium salt prepared from the mother-liquors was of almost equal purity.

Fractional hydrolysis. Lohmann's method of separation of the esters by fractional hydrolysis was also tried but, as in his experiments, with only partial success. For this attempt we used a sample of hexosemonophosphate that had already been purified by recrystallisation of the brucine salt from 20 % ethyl alcohol and contained 80 % of the aldose derivative. The barium salt was hydrolysed with *N* H_2SO_4 for 6 hours at 100°, 14 % of the phosphate being set free. The residual monophosphate now contained 84 % of the aldose ester. After a further 6 hours in *N* H_2SO_4 at 100° the proportion of aldose ester had increased to 88 % and rose to 91 % after a third hydrolysis of similar duration. During the 18 hours 35 % of the original ester had been hydrolysed.

Other components of hexosemonophosphoric ester.

The dual nature of the platelet fractions obtained during the crystallisation of the brucine salt from 20 % ethyl alcohol was confirmed by submitting the barium salts derived from these fractions to bromine oxidation. The salts, dissolved in water, were treated with bromine and barium carbonate at room temperature. The flask was shaken at intervals during 24 hours and the excess of bromine then removed by aeration. The filtered solution was made acid to methyl orange and treated with 4 times its volume of alcohol by which the acid salt of the oxidation product (phosphohexonic acid) was precipitated. The filtrate was neutralised to phenolphthalein with baryta and the precipitated barium hexosemonophosphate filtered off, purified by solution in 10 % alcohol and reprecipitation, and analysed. After a second treatment with bromine the purified barium salt had a very low iodimetric value and specific rotation, and was very similar in most respects to Neuberg's fructosemonophosphate. For the purposes of comparison a specimen of the latter ester was prepared by partial hydrolysis of hexosediphosphate which had been very carefully purified by precipitation of the barium salt from its aqueous solution by heating the latter to 75°. This purification was very essential since the diphosphate is likely to be contaminated with small amounts of aldosemonophosphate carried down as the double barium salt [*v.* Robison and Morgan, 1930]. Such aldosemonophosphate is not appreciably hydrolysed during the short treatment with acid and therefore becomes concentrated in the fructosemonophosphate produced. The chief differences between the ester derived from the platelet fraction and the Neuberg ester lay in the lower Hagedorn and Jensen reducing power of the former, which may indicate that it was contaminated with the unknown ester referred to below.

The final mother-liquors from the crystallisation of the brucine salts in 20 % alcohol still contained a considerable amount of phosphoric ester, very soluble in this solvent and even in a much higher concentration of alcohol. The analyses of the barium salts prepared from these mother-liquors indicated the presence of some unknown ester since the analytical data could not be

reconciled with any possible mixture of the four known esters. The specific rotations of these salts were higher than that of the aldosemonophosphate, but this might be due to the presence of a very small proportion of trehalosemonophosphate. Whether these salts contain the new ester in small or large proportion cannot be decided until it has been isolated and its properties investigated. In spite of much work this has not yet been achieved. A comparison of the Hagedorn and Jensen values found for the original hexosemonophosphate (*cf.* Table I, salts 1–4) with those of the pure aldose and ketose esters suggests, however, that the unknown ester is present in significant amounts.

Analyses of the various barium salts obtained in these operations and referred to in the above paragraphs are shown in Table I. One of the original specimens of hexosemonophosphate prepared 10 years ago from glucose [Robison, 1922, Table II, No. 7], has also been analysed by the micro-methods now employed and the results are included in Table I. Details of these methods have already been given, but some notes may be added here. The estimations were carried out on the substance dried over sulphuric acid, the moisture being separately estimated in Pregl's micro-drying apparatus. The results are calculated for the anhydrous salt. The values quoted for "fructose" were found by estimating the colour developed with the Selivanoff reagent and do not represent the actual percentage of this hexose in the sample. Thus, the value for barium fructosemonophosphate is only half the percentage of hexose in the salt.

The estimation of reducing power by the Hagedorn and Jensen method was carried out with addition of 0·5 cc. $N/2$ sodium hydroxide to each tube. Without this addition the curve of reducing power for these esters deviates so widely from the curve determined for glucose that very inconsistent values are obtained by reference to the glucose table. Even with this extra alkali the percentage reducing power is found to vary a little according to the amount of ester taken. For both glucose and fructosemonophosphates the values lie between 35 % and 37 %, that is, the reducing power is about 80 % of that of the unsubstituted hexose.

In the iodimetric estimations, carried out by the method of Macleod and Robison [1929], it was found that the aldosemonophosphate was even more sensitive than glucose to various conditions, particularly to the concentration of alkali. Low results were obtained unless the iodine was in very large excess or if the amount of 5 % sodium carbonate solution was increased from 0·2 cc. to 0·4 cc., an amount shown to be permissible in the estimation of glucose.

The specific rotation of barium aldosemonophosphate varies slightly with the concentration of the salt and still more with changes in p_H of the solution. Thus, the value of $[\alpha]_{5461}$ for salt No. 11 in 0·5 % solution (p_H *circ.* 8·3) was + 19·6°, at p_H 9·8 it was + 18·0°, while at p_H 5·8 it was + 23·3°. The free acid corresponding to this salt had $[\alpha]_{5461}$ + 40·0°, but in 0·07 N H_2SO_4 this

became + 43·6°. These differences are possibly due to changes in the equilibrium between the un-ionised ester and its ions.

Table I. *Analyses of barium salts of hexosemonophosphoric acids obtained from the products of fermentation by yeast juice.*

No. of salt	Source of the barium salt	P %	Fructose (Seliwanoff) %	Reducing power as glucose (%) H. and J.	Reducing power as glucose (%) Iodine	Aldose-monophosphate %	$[\alpha]_{5461}^{20°}$	$[\alpha]_{5461}^{20°}$ of free acid
1	"Hexosemonophosphate" [Robison, 1922]	7·85	6	30·0	25·2	55	+14·4°	+29·5°
2	Soluble-B fraction	7·37	9	27·4	21·1	46	+11·3°	—
3	,,	7·45	—	30·8*	27·7	61	+15·0°	—
4	Soluble-B after further purification by mercuric acetate and basic lead precipitation	7·90	5	31·9	23·6	52	+16·3°	—
5	Brucine salt after repeated fractional crystallisation from 20 % ethyl alcohol. Hard, translucent needles	7·80	—	31·8*	41·4	91	+21·0°	—
6	Brucine salt recrystallised 4 times from 20 % ethyl alcohol. Hard needles	7·84	0·5	34·9	36·1	79	+17·9°	—
7	Brucine salt recrystallised 4 times from 20 % ethyl alcohol, 3 times from 90 % methyl alcohol. Opaque, silky needles	7·86	0·5	36·1	41·0	90	+19·2°	—
8	Brucine salt recrystallised twice from 20 % ethyl alcohol, 4 times from 95 % methyl alcohol, 3 times from absolute methyl alcohol. Opaque, silky needles	**7·86**	**0·5**	**35·5**	**45·7**	**100**	+20·6° (c=0·84 %) +21·2° (c=8·4 %)	+41·4° (c=0·74 %)
9	Mother-liquors in final recrystallisation of above brucine salt	7·87	0·5	35·3	44·9	99	+20·1° (c=1·4 %)	—
10	Brucine salt recrystallised from 20 % ethyl alcohol	7·90	2·4	35·1	36·6	80	+16·2°	—
11	Residual salt after 18 hrs. hydrolysis of No. 10, with N H_2SO_4 at 100°	7·90	? 0	34·8	41·4	91	+19·6°	+40·0°
12	Brucine salt fractionally crystallised from 20 % ethyl alcohol. Hexagonal plates	7·76	—	25·4	18·9	41	+ 8·2°	—
13	Residual salt after oxidation of No. 12 with bromine and removal of phosphohexonate	7·75	22	27·6	2·6	6	+ 2·1°	—
14	Residual salt after oxidation of another similar fraction	7·60	20	29·0	1·3	3	+ 3·3°	—
15	Fructosemonophosphate (Neuberg) prepared from highly purified hexosediphosphate	7·80	22	36·2	3·0	7	+ 0·7°	—
16	Final mother-liquors in the recrystallisation of brucine salts	7·05 6·90	6·8 5·7	25·3 25·0	16·7 21·1	37 46	+21·4° +22·2°	— —

$C_6H_{11}O_8PO_4Ba$ requires P = 7·85 %; $C_6H_{12}O_6$ = 45·6 %.

* Determined without addition of NaOH.

Osazones of hexosemonophosphoric esters.

The osazone of hexosemonophosphoric ester was originally described [Robison, 1922] as melting at 139°, and it was concluded that this osazone was not identical with that prepared by Young [1911] from Harden and Young's fructosediphosphoric ester. Should the aldose ester prove to be a derivative of glucose, or of mannose, the non-identity of the osazones would show that the phosphoric acid group is not in the same position in the two esters. The demonstration of the composite nature of the original hexosemonophosphate had, however, thrown doubt on the purity of the osazone

prepared from it and a re-examination of this point was, therefore, necessary. Osazones were prepared from the pure aldosemonophosphate (No. 8, Table I) and from the Neuberg fructosemonophosphate (No. 15) prepared from hexosediphosphate. Solutions of the free acids, prepared from the barium salts, were treated with as much phenylhydrazine as they would dissolve before adding the calculated amount of the base in acetic acid. The solutions were heated in a boiling water-bath for short periods ($\frac{1}{2}$ hour), cooled to 0°, filtered and again heated. The osazones were recrystallised by dissolving in boiling alcohol and adding chloroform to the filtered solution. For the determination of the melting points a short thermometer, with the mercury thread completely immersed in the bath, was used and the melting points are, on this account, higher than that given by Young (151–152°). The tube containing the osazone was introduced when the temperature of the bath was 4° below the melting point and was rising at the rate of 6–8° per minute. The osazones from the aldosemonophosphate and from fructosemonophosphate both melted with decomposition at 154–154·5° and an intimate mixture of the two melted at the same temperature. The yield of osazone obtained from the pure aldosemonophosphate was equal to 48 % of the weight theoretically possible. More than half of this yield was obtained from the first period of heating and the melting point of this sample before recrystallisation was identical with that of the recrystallised osazone. The successive products melted within one or two degrees of this temperature.

Analyses. The P content corresponded with that of a phenylhydrazine salt of the osazone of hexosemonophosphoric acid.

Osazone from aldosemonophosphate gave P = 5·63 %.
,, fructosemonophosphate (Neuberg) gave P = 5·61 %.
Calculated for $C_{24}H_{31}O_7N_6P$ = 5·68 %.

The specific rotation of the osazone from aldosemonophosphate was determined in pyridine-alcohol (2:3).

$[\alpha]^{21°}_{5461}$ − 60° after 15 min., − 38° after 85 min., − 35° after 24 hrs.

Neuberg and Reinfurth [1924] give $[\alpha]_D$ − 51° after 15 minutes and − 36° after 1 hour (equilibrium) for the phenylhydrazine salt of the osazone of fructosemonophosphate and also for that obtained from hexosediphosphate.

Further evidence was obtained of the identity of the two osazones by measuring the rate of hydrolysis in *N* H_2SO_4 at 100°. The results are shown below.

	Aldosemonophosphoric ester	Fructosemonophosphoric ester	Osazone of aldosemonophosphoric ester	Osazone of fructosemonophosphoric ester
		%	%	%
Hydrolysis in 1 hour	—	—	47	44
Hydrolysis in 2 hours	0	41	80	75

The two osazones were hydrolysed at approximately equal rates, which were twice as great as that for fructosemonophosphoric ester. The hydrolysis

of aldosemonophosphoric ester was too small to be estimated with the quantities taken.

An osazone was also prepared from the ketose component of the platelet fraction (No. 14) and this also, after recrystallisation, melted at 154° and at the same temperature when mixed with the osazone of fructosemonophosphoric acid.

Hydrolysis of aldosemonophosphoric ester.

Hydrolysis by acids. The hydrolysis of aldosemonophosphoric ester was studied by heating a 0·03 *M* solution of the free acid at 100° and also by heating the ester, in similar concentration, with 0·1 *N* and with *N* H_2SO_4 at 100°. The results of two experiments are shown in Fig. 1, the actual rates

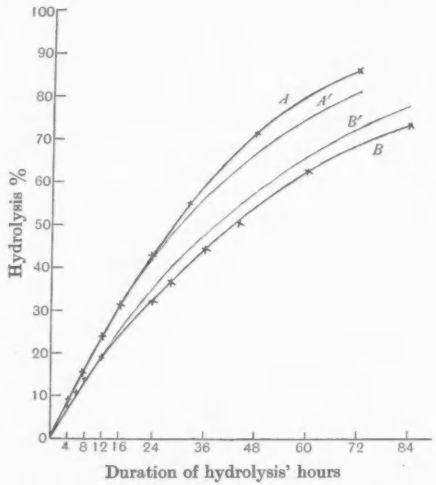

Fig. 1. *A*. Autolysis of 0·03 *M* aldosemonophosphoric ester at 100°.
A'. $k = 0{\cdot}167 \times 10^{-3}$. *B'*. $k = 0{\cdot}13 \times 10^{-3}$.
B. Hydrolysis of 0·03 *M* aldosemonophosphoric ester in *N* H_2SO_4 at 100°.

of hydrolysis being shown by the thick lines while the thin lines are theoretical curves of unimolecular equations drawn for the velocity constants calculated from the results of the first 16 hours' hydrolysis ($k = 1/t \log a/(a - x)$, t being time in minutes). No sharp deviation from these theoretical curves is shown in either case, but a gradual, though slight, falling off in the rate is shown for the hydrolysis in *N* H_2SO_4, while a slight increase is shown in the rate of hydrolysis of the free ester alone. The results appear to support the view that we are dealing with a homogeneous ester. It was very surprising, however, to find that the hydrolysis proceeded more rapidly in the solution containing aldosemonophosphoric acid alone than in presence of *N* H_2SO_4, but this fact was confirmed by a repetition of both experiments. Further, with 0·1 *N* H_2SO_4

the rate of hydrolysis was very slightly higher (66 % in 60 hours) than with *N* acid (63 % in 60 hours). Is it possible that these results are due to the presence of two ring systems, one of which, favoured by the presence of sulphuric acid, hinders the removal of the phosphoric acid group? The increased specific rotation of the free ester in presence of sulphuric acid might possibly be connected with the formation of this ring and the sensitiveness of the ester towards alkali, noted in reference to the iodimetric estimation, may also be related to such an equilibrium (*cf.* Lohmann's [1928] suggestion quoted on p. 323 of this paper).

The sugars produced from aldosemonophosphoric ester by hydrolysis with 0·1 *N* H_2SO_4 and heating the solution of the free acid alone were isolated and examined. The solutions were neutralised with baryta and treated with 4 times their volume of alcohol. The precipitated barium salts were removed by filtration and thoroughly washed with 80 % alcohol. The filtrate was evaporated on the water-bath to a small bulk and then in an evacuated desiccator over sulphuric acid. The results of the examination of the products are shown in Table II. The properties of the sugar obtained from hydrolysis with 0·1 *N* H_2SO_4 corresponded very closely with those of glucose. That obtained from the partial hydrolysis of the ester without H_2SO_4 had a somewhat lower specific rotation and was less pure. A control experiment was carried out by heating a solution of glucose for 60 hours with 0·1 *N* H_2SO_4 and then recovering the sugar in the manner described above; it is seen from the results that very little decomposition or alteration of the glucose occurred.

Hydrolysis by bone phosphatase. Two experiments were carried out on the hydrolysis of the aldosemonophosphoric ester by the bone phosphatase. 0·4 g. of the pure barium salt was converted into the free acid and the solution (15 cc.) treated with sodium hydroxide to bring the p_H to 7·0. A purified preparation of bone phosphatase (50 mg.) was added and the solution kept at room temperature in presence of chloroform, the p_H being checked and the liberated inorganic phosphate estimated at intervals. Hydrolysis was practically complete in 4 days. The solution was evaporated to dryness *in vacuo* over sulphuric acid and the residue was extracted repeatedly with hot 80 % ethyl alcohol. The filtrate was evaporated, first on the water-bath and finally in an evacuated desiccator and the residue dissolved in 30 cc. water for examination. The hydrolysis was carried out in neutral solution and at room temperature in order to minimise the risk of the Lobry de Bruyn transformation taking place. The slower rate of hydrolysis at room temperature is compensated for by the fact that no destruction of enzyme occurs (as it does at 37°) so that hydrolysis will continue smoothly even during several weeks. In spite of these mild conditions it was evident that the sugar product contained a considerable proportion of ketose. The analytical results and the preparation of an osazone, which was identified by its melting point (206°) with glucosazone, justified the conclusion that the product contained glucose and fructose. Whether mannose was also present is uncertain, but an attempt to obtain the

phenylhydrazone was not successful. This transformation of the sugar component of monophosphoric ester on hydrolysis with phosphatase is in agreement with the results found by Martland and Robison [1929] for the enzymic hydrolysis of hexosediphosphoric ester. Control experiments carried out in a similar manner with bone phosphatase + distilled water, and with phosphatase + 0·5 % glucose solution showed that only a very small amount of reducing and optically active material could be derived from the phosphatase and that glucose itself suffered no transformation under the conditions of the hydrolysis.

Table II.

Method of hydrolysis	Duration of hydrolysis hrs.	Hydrolysis %	Wt. of hexose equivalent to P liberated mg.	Wt. of syrup dried over H_2SO_4 mg.	Reducing power as glucose (mg.) H. and J.	Reducing power as glucose (mg.) Iodine	Fructose (Seliwanoff) mg.	$[\alpha]^{20°}_{5461}$ calculated on the H. and J. value
1. 0·1 N H_2SO_4 at 100°	60	66	86	83	62	64	6	+60·5°
2. Free acid alone at 100°	44	73	92	118	88	92	7	+54°
3. Bone phosphatase at room temperature and p_H 7·0	96	99	144	155	133	116	24	+33°
4. Bone phosphatase at room temperature and p_H 7·0 (Results of 3 and 4 corrected for phosphatase blank)	80	97	82	—	82	67	13	+29°
Controls:			Wt. of glucose taken					
20 cc. 0·5 % glucose solution +80 cc. alcohol evaporated to dryness and extracted with 80 % alcohol			100	100	96	97	1	+63°
27 cc. 0·5 % glucose solution heated with 0·1 N H_2SO_4 60 hrs.; neutralised and treated as above			135	155	125	133	3	+62°
30 cc. 0·5 % glucose solution +50 mg. bone phosphatase evaporated to dryness in desiccator and extracted with 80 % alcohol. (Results corrected for phosphatase blank)			150	160	143	147	2	+63°
30 cc. H_2O +50 mg. bone phosphatase evaporated to dryness in desiccator and extracted with 80 % alcohol			0	5	0·4	1·3	0	α_{5461} −0·04° (l = 4 dm.)

Preparation of a phosphohexonic acid from hexosemonophosphoric ester.

A preparation of hexosemonophosphate, which had been purified by recrystallisation of the brucine salt from 20 % ethyl alcohol and contained about 80 % of the aldose ester, was oxidised with bromine in order to convert this ester into the corresponding phosphohexonic acid. To 10 g. of the barium salt, dissolved in 80 cc. water, 1·4 cc. bromine and 12 g. barium carbonate were added, a further 1·4 cc. bromine being added after 24 hours. The solution was kept at room temperature and shaken at intervals. Iodimetric estimations carried out on 0·2 cc. portions, after removal of the bromine, indicated that oxidation was complete after 48 hours. The excess of bromine was removed by aeration and the barium carbonate by filtration. The filtrate (p_H 3·5) was poured into 5 times its volume of alcohol upon which the acid salt of the phosphohexonate was precipitated. This was filtered off, washed thoroughly with 85 % alcohol and dried. It was then dissolved in 20 cc. water with the aid of 2 cc. N HCl and, after filtration, treated with a solution of barium

hydroxide till pink to phenolphthalein. The precipitate was filtered off, washed with water and absolute alcohol and dried over sulphuric acid *in vacuo*. It was non-reducing and gave a negative Selivanoff reaction.

P found 6·42 %; calculated for $(C_6H_{10}O_{10}P)_2Ba_3$, P = 6·48 %.

$[\alpha]_{5461}^{21°} = -1·5°$.

Solubility in water at 100°. 100 cc. solution contained 0·71 g.

The acid barium salt was prepared by dissolving 1 g. of the neutral salt in sufficient dilute hydrochloric acid to make the solution just acid to methyl orange and adding 5 times the volume of alcohol. The precipitate was filtered off, washed thoroughly with 85 % and absolute alcohol and dried *in vacuo*.

P found 7·40 %; calculated for $C_6H_{11}O_{10}PBa$, P = 7·54 %.

$[\alpha]_{5461}^{21°} = +0·2°$.

A solution of the free phosphohexonic acid was prepared by decomposing 0·25 g. of the barium salt with the exact amount of sulphuric acid and removing the barium sulphate by centrifuging. The change in rotation due to lactone formation is shown below, the times being measured from the addition of the sulphuric acid. After heating to 70° the solution was rapidly cooled.

Time	$\alpha_{5461}^{21°}$ (l=4 dm.)	$[\alpha]_{5461}^{21°}$
10 minutes at 21°	0	0
2 hours at 21°	+0·03°	+ 2°
18 hours at 21°	+0·08°	+ 5°
1 hour at 70°	+0·26°	+16°
2 hours at 70°	+0·285°	+18°

The solution was finally heated for an hour at 70° with 0·1*N* HCl and cooled rapidly when the $[\alpha]_{5461}$ was + 21°. The yield of phosphohexonate was equivalent to 62 % of the original hexosephosphate, but a further quantity of less pure salt was recovered from the first acid-alcohol filtrate together with a small amount of fructosemonophosphate.

Preparation of hexonic acid by hydrolysis of the phosphohexonic acid with bone phosphatase.

The hydrolysis of the phosphohexonate was carried out by means of bone phosphatase in two separate experiments.

1st method. 1 g. of the neutral barium salt in 20 cc. water was treated with 0·5 g. of purified phosphatase at 37° and p_H 8·6–8·8. The flask was shaken at frequent intervals and barium hydroxide solution added as required to maintain the stated p_H. Hydrolysis was complete in about 9 hours and the solution was then heated at 100° and filtered. The residue was well washed with hot water and the filtrate and washings were concentrated on the water-bath. The barium hexonate was converted into the calcium salt by treatment with a solution of calcium sulphate. The filtered solution was again concentrated and poured into absolute alcohol to precipitate the salt, which was then filtered off, washed with alcohol and dried *in vacuo*. The salt was free from phosphate but estimation of the calcium indicated that it contained a little calcium sulphate which could not be easily removed.

2nd method. 1·35 g. barium phosphohexonate was treated with the exact quantity of sulphuric acid required to precipitate the barium and the sulphate removed by centrifuging and thoroughly washed. The acid solution was heated with pure calcium carbonate, filtered and treated with sufficient calcium hydroxide to raise the p_H to 6·0. (The addition of more calcium hydroxide caused the salt to be precipitated.) After cooling the solution was treated with 0·5 g. of purified bone phosphatase and a few drops of chloroform and was left in a stoppered flask at room temperature. Each day the p_H was noted but required no adjustment and the progress of the hydrolysis was determined by estimating the inorganic phosphate in 0·2 cc. Hydrolysis was complete in 4 days, after which the solution was heated on the water-bath and treated with calcium hydroxide until pink to phenolphthalein. It was filtered and the residue washed with boiling water. The filtrate was concentrated on the water-bath and poured into absolute alcohol; the precipitate was filtered, washed with alcohol, dried, and purified by solution in water and reprecipitation with alcohol. The salt was free from phosphorus and did not reduce hypoiodite. By the Hagedorn and Jensen method a very slight reduction (1 %) was found.

Ca found 9·4 %; calculated for $(C_6H_{11}O_7)_2Ca$, 9·30 %.
$[\alpha]^{20°}_{5461}$ + 6·8° (l = 4 dm., c = 0·8 %).

The recorded values for the $[\alpha]_D$ of calcium gluconate vary between + 6° and + 10·5°.

A solution of the free acid was produced by treating the calcium salt with the calculated quantity of oxalic acid and filtering. The change in rotation due to lactone formation is shown below.

Time	$\alpha^{20°}_{5461}$ (l=4 dm.)	$[\alpha]^{20°}_{5461}$
20 minutes at room temperature	−0·08°	− 2·9°
18 hours at room temperature	+0·22°	+ 8·6°
2 hours at 70°	+0·54°	+21·0°
3 hours at 70°	+0·59°	+22·9°
In 0·1 *N* HCl 2 hours at 70°	+0·71°	+27·8°
3 hours at 70°	+0·71°	+27·8°
66 hours at room temperature	+0·52°	+20·4°

The results agree with the recorded values for *d*-gluconic acid if the factor 1·18 is used to convert $[\alpha]_D$ into $[\alpha]_{5461}$. Rehorst [1928] gives the following values for $[\alpha]^{20°}_D$ in 2·8 % aqueous solution: after 5 mins. − 6·72°; after 20 mins. − 2·75°; after 24 hrs. + 7·02°; after 5 days + 11·9°. In Tollen's "Handbuch der Kohlenhydrate" (3rd edition) the equilibrium mixture obtained on heating the free acid is stated to have $[\alpha]_D$ + 23·4°, which, multiplied by 1·18 gives $[\alpha]_{5461}$ + 27·6°.

Oxidation of aldosemonophosphoric acid with nitric acid.

It was considered that if the phosphoric acid group is in position 3 of the aldose molecule, or in any position other than 6, it should be possible to obtain

a phosphodicarboxylic acid by oxidation of the ester itself or of the phosphohexonate with nitric acid. Accordingly, a number of attempts to prepare such a compound were made by treating the pure aldosemonophosphate and phosphohexonate described above with nitric acid of different concentrations, at different temperatures, and for various periods of time. The phosphohexonate manifested a considerable resistance to further oxidation under mild conditions and when oxidation did occur it was invariably accompanied by hydrolysis. The products obtained from this and from the aldose ester were always mixtures containing dicarboxylic acids, but in no case could the analytical results be interpreted as providing evidence of the presence of a dicarboxylic acid in which the phosphoric group was still present.

Discussion.

The results we have described lead us to conclude that one, and possibly the chief, constituent of hexosemonophosphoric ester of fermentation has been isolated in pure homogeneous condition and that this compound has the constitution of an aldosemonophosphoric ester. The properties of the sugar obtained from this ester by acid hydrolysis as well as those of the hexonic acid formed from it by oxidation with bromine followed by enzymic removal of the phosphate group, provide evidence of the identity of the aldose with *d*-glucose. Hydrolysis of the ester by phosphatase has been shown to involve partial transformation of the hexose as already demonstrated for the hydrolysis of fructosediphosphate. This transformation is in itself of interest but the properties of the sugar produced obviously cannot be accepted as proving the nature of the aldose present in the ester.

For the position of the phosphoric acid group in this ester 2 is excluded by the formation of a phosphohexosazone, while the formation of both stable and unstable methylhexosides [King and Morgan, 1929; King, McLaughlin and Morgan, 1931] very probably excludes positions 4 and 5. Position 6 appeared at first to be excluded by the differences between the melting points of the osazone originally obtained from hexosemonophosphoric ester and of the osazone prepared by Young from fructosediphosphoric ester and later by Neuberg and Reinfurth [1924] from fructosemonophosphoric ester. This difficulty has disappeared since the osazone now prepared from the pure aldosemonophosphate has been found to have the same melting point and rate of hydrolysis as the osazone of fructosemonophosphoric ester in which the phosphoric acid group is considered to occupy position 6.

Our failure to obtain a phosphodicarboxylic acid by oxidation of the aldosemonophosphoric ester with nitric acid further supports the view that the ester is a glucose-6-phosphate. The properties of the pure ester do not, however, entirely agree with those of any of the synthetic glucosephosphates which have been prepared by a number of workers [Komatsu and Nodzu, 1924; Nodzu, 1926; Raymond and Levene, 1929; Josephson and Proffe, 1930; Levene and Raymond, 1930]. In their last paper Levene and Raymond [1930]

report a new examination of the ester synthesised from di-*iso*propylideneglucose and conclude, in agreement with Josephson and Proffe, that this ester is glucose-3-phosphate, and that it differs from the hexosemonophosphate of fermentation in its specific rotation and its reaction with phenylhydrazine, with which it forms a 3:6-anhydrohexosazone. They also find that its rate of fermentation is much slower than that of the natural ester. Levene and Raymond have further synthesised a phosphoric ester from *iso*propylideneglucose which they consider to be glucose-6-phosphate and have shown that it gives a phosphohexosazone identical with that obtained from the Harden and Young and Neuberg esters, and that its rate of fermentation is identical with that of hexosemonophosphate. The specific rotations given for this synthetic ester and its barium salt do not, however, agree with those of the pure aldosemonophosphoric ester described in the present communication.

By multiplying Levene and Raymond's values for $[\alpha]_D$ of their synthetic esters by the factor 1·18 the following values for $[\alpha]_{5461}$ are obtained for comparison with those of the aldose ester here described:

	$[\alpha]_{5461}$ of barium salt	$[\alpha]_{5461}$ of free acid
Synthetic ester from di-*iso*propylideneglucose (glucose-3-phosphate)	+31·3°	+46·6°
Synthetic ester from *iso*propylideneglucose (glucose-6-phosphate)	+15·3°	+30·6°
Pure aldosemonophosphoric ester	+21·2°	+41·4°

Although Levene and Raymond state that the ester prepared from monoacetone glucose may not have been quite pure, the differences in the specific rotations are sufficiently great to cause hesitation in concluding that this synthetic ester is identical with the aldosemonophosphate of fermentation.

Apart from this discrepancy all the evidence at present available goes to show that the natural ester whose isolation and properties we have described is glucose-6-phosphate. From the iodimetric estimations it would appear that this ester cannot normally form more than about 50–70 % of the hexosemonophosphate isolated from the products of fermentation, while it may well be that part of this percentage represents other esters, *e.g.* a disaccharide-diphosphate, which would account for the low Hagedorn and Jensen reducing power of some of the other fractions isolated during the investigation of the mixed hexosemonophosphate. That the latter contains also the Neuberg fructosemonophosphate seems to be certain, but whether this ester accounts for the whole of the non-aldose fraction, as estimated iodimetrically, will not be settled until the properties of the unknown ester are determined.

Trehalosemonophosphoric ester has not been isolated from these yeast juice products, although the high specific rotation of certain small fractions obtained in the crystallisation of the brucine salt might be accounted for by its presence. Monophosphoric esters (not yet obtained in pure condition) of higher specific rotation than that of aldosemonophosphate have been described by Euler, Myrbäck and Runehjelm [1928] and by I. S. Neuberg

and Ostendorf [1930] (from the products of fermentation of mannose by fresh yeast and toluene), but in the case of these compounds also the presence of trehalosemonophosphate in small proportion might possibly account for the high specific rotation recorded by the authors. It does not, however, necessarily follow that this is the true explanation and further information with regard to these esters will be awaited with interest.

Summary.

1. An aldosemonophosphoric ester has been isolated in pure condition from the hexosemonophosphoric ester produced by fermentation of hexoses with yeast juice.

2. The $[\alpha]^{20°}_{5461}$ of the free ester is + 41·4°, and of the barium salt + 21·2° ($c = 8{\cdot}4$ %). The reducing power of the ester by an iodimetric method is equal to that of the equivalent amount of glucose. Its reducing power by the Hagedorn and Jensen method is 80 % of that of glucose. The Selivanoff reaction is not appreciably greater than that given by glucose.

3. The phenylhydrazine salt of the osazone of aldosemonophosphoric ester has been prepared and shown to be identical both by its melting point and its rate of hydrolysis with the corresponding salt of the osazone of fructosemonophosphoric ester; M.P. 154–154·5°.

4. The ester is very resistant to hydrolysis by acids at 100°. Hydrolysis proceeds more rapidly when the free aldosemonophosphoric acid is heated alone than in presence of sulphuric acid. The sugar product of acid hydrolysis has the character of *d*-glucose.

5. Hydrolysis by bone phosphatase proceeds rapidly at room temperature and p_H 7·0, but the sugar product contains both glucose and fructose.

6. Oxidation of the aldosemonophosphoric ester with bromine yields a phosphohexonic acid from which gluconic acid has been obtained by hydrolysis with bone phosphatase.

7. Oxidation with nitric acid failed to yield any evidence of the production of a dicarboxylic acid containing a phosphoric acid group.

8. These experiments in conjunction with those reported by King, McLaughlin and Morgan point to the constitution of the ester being that of a glucose-6-phosphate.

9. The specific rotation of the free ester and of its barium salt differ considerably from those of the synthetic ester prepared by Levene and Raymond from *iso*propylideneglucose, and considered by them to be glucose-6-phosphate.

10. A ketosemonophosphoric ester similar in most respects to Neuberg's fructosemonophosphoric ester has also been isolated from the fermentation products, while evidence has been obtained of the presence of another ester as yet unidentified.

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XLI. STUDIES IN BLOOD GLYCOLYSIS.
PRELIMINARY OBSERVATIONS.

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This study was undertaken to determine the factors concerned in glycolysis in blood incubated at 37°. It is clear from the work of a number of observers—Schmitz and Glover [1927], Stammers [1926] and Chahovitch [1926]—that (1) sterile blood loses its sugar if kept at room or body temperature; (2) the rate of glycolysis varies in different species.

Glycolysis was studied under the following conditions, *viz.* (1) in whole blood after varying periods of starvation and after feeding, (2) in laked corpuscles, (3) in serum, (4) in cyanide blood, (5) in washed erythrocytes, (6) in washed erythrocytes and glucose solution.

Method.

Our observations have been confined mainly to dog's blood. About 2 cc. of blood were drawn from one of the leg veins and incubated at 37° for a variable period; samples were taken therefrom before incubation and at intervals of 30, 60 and 120 minutes for sugar estimation by MacLean's method. During the process of taking blood asepsis was observed although it was found that this precaution was not necessary at any rate for incubation periods not extending beyond 3 hours.

It will be seen on reference to Table I that the rate of glycolysis is greatest during the first hourly period of incubation; it is greater in blood samples taken about 2 hours after the ingestion of food than after a day's starvation and it decreases considerably after 2 days' starvation.

Effect of cyanide.

To determine whether glycolysis in blood is largely or entirely an oxidative process, blood was mixed with different quantities of cyanide solution in normal saline as follows: 2 cc. blood samples were taken and mixed with 1 cc., 0·5 cc. and 0·2 cc. of 0·3 % KCN solution and the total volume was made up to 3 cc. with normal saline. A control containing 2 cc. blood and 1 cc. saline was also set up. Preliminary glucose estimations were made and the samples were incubated at 37° in small test-tubes. It will be noticed

(Table II) that the quantity of cyanide used has no effect on glycolysis in whole blood and there is no relationship between the quantity of cyanide used and the degree of glycolysis.

Table I.

Blood-sugar in mg./100 cc.	Rate of glycolysis in mg. glucose per 100 cc.		
	½ hour	1 hour	2 hours
(*a*) Two hours after food:			
108·5	19	28	48·5
104·5	19·5	42·5	53·5
97	5	24	35
111	9	22	37
Average	13·1	29	43·5
(*b*) Starvation (1 day):			
87	13	24	39
105·5	8·5	25	31·5
101	10	22·5	38
102	—	—	33
Average	10·5	23·8	35·4
(*c*) Starvation (2 days):			
71·5	4	11	13·5
73	13	15	20
90·5	2·5	8	25
Average	6·5	11·3	19·5

Table II.

	Composition of incubated blood sample			Blood-sugar in mg./100 cc.	Rate of glycolysis in mg. glucose per 100 cc.	
	Blood cc.	KCN cc.	Saline cc.		1 hour	24 hours
(1)	2	1	—	82	10	34
(2)	2	0·5	0·5	82	10	22
(3)	2	0·2	0·8	82	8	22
(4)	2	—	1	60	—	30

Table III.

Composition of incubated sample	Sugar in mg./100 cc.	Rate of glycolysis in mg. glucose per 100 cc.	
		1 hour	24 hours
(*a*) Serum	114	2	—
	113	5·5	—
	115	4	—
	111	6	—
	Average	4·4	
(*b*) 2 cc. serum + 1 cc. erythrocytes	104	20	—
(*c*) 2 cc. glucose solution in saline + 1 cc. erythrocytes	172	8	65
(*d*) 2 cc. glucose solution in dist. water + 1 cc. erythrocytes (laked corpuscles)	172	6·5	13

Observations were then made on glycolysis in serum, in laked blood and in glucose solutions in the presence of intact washed erythrocytes, to note the rate of glycolysis and the part played by the corpuscles, if any, in the pheno-

menon. Glycolysis was found to occur to a slight degree in the course of 1 hour in incubated serum and in laked corpuscles in contrast to the findings of Katyama [1926] (Table III, *a*, *d*) and to be greater in serum or glucose solutions in the presence of intact washed erythrocytes (Table III, *b*, *c*).

The results obtained above in (*c*) and (*d*) are not affected by the substitution of glucose dissolved in serum for an ordinary glucose solution.

Discussion.

In connection with the increased glycolysis after the ingestion of food as compared with the amount of glycolysis in the blood from a starving animal, three possible factors at least must be taken into consideration.

(1) Increased percentage of sugar in the blood.

(2) Increased amount of glycolytic substance in the blood.

(3) Effect of increased amount of insulin released (following the ingestion of food) on the tissue cells leading to an increased liberation of the substance concerned with glycolysis.

Observations on these points are being continued.

Further, the question arises as to the factors concerned in glycolysis and the nature of the process of glycolysis. It might be supposed that the latter is one of oxidation but our observations confirm those of Abraham and Altmann [1927] in that cyanide, which inhibits oxidising enzymes, has no effect on the glycolytic power of blood.

From the result that glycolysis proceeds most rapidly in whole blood as compared with the rate of glycolysis in serum, glucose solutions + intact erythrocytes and glucose solutions + laked erythrocytes, it may be inferred that there are factors present in both serum and erythrocytes which are essential for the increased glycolysis observed. Further work is being done to elucidate this point.

Summary.

1. Starvation decreases and the administration of food increases the rate of glycolysis in blood.

2. The factors responsible for glycolysis in blood are present in both erythrocytes and serum.

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XLII. THE ADDITION COMPOUND THEORY OF ENZYME ACTION.

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(*Received December 24th, 1930.*)

1. Introduction.

In 1929 the writer, as a result of a study of three enzymes in *B. coli communis* which act on fumaric acid, propounded "the rather attractive hypothesis that one of the conditions for a reaction to occur at these enzymes is that all the substrates shall be combined there together—succinic acid and methylene blue for succinoxidase action, fumaric acid and ammonia with aspartase, and fumaric acid and water with fumarase" [Woolf, 1929]. Since that time, further experimental findings, coupled with a thorough study of the work of others, have led the writer to develop this hypothesis into a definite theory of enzyme action, which it is the object of this paper to describe.

According to the view about to be put forward, the meaning of the term substrate is widened to include all the molecules participating in the catalysed reaction, including, for example, water in a hydrolysis, and the methylene blue or other hydrogen acceptor in a dehydrogenase action. The addition compound theory may then be stated as follows. An enzyme is a definite chemical compound which is able to form an unstable addition compound with all its substrates, each at its own specific combining group in the enzyme molecule. The process of catalysis then consists of a series of tautomeric changes in the enzyme-substrate complex, as a result of which, in a certain proportion of cases, the complex is able to dissociate into free enzyme plus the products of the catalysed reaction.

The line of reasoning on which this theory is based falls naturally into two sections, first an examination of all the known data of enzyme kinetics, and the induction from them of general laws, followed by the deduction from these laws of the probable mechanism of the catalytic process. A complete presentation of the first section is obviously impossible in this place. The full evidence for the conclusions given below will be set out, however, in a review by the writer on "Enzyme Kinetics," which is now in course of preparation for *Biological Reviews*.

2. Enzyme kinetics.

The enzyme-substrate compound.

The hypothesis that the enzyme and substrate unite stoichiometrically was first put forward explicitly by Michaelis and Menten [1913], who deduced from it the well-known equation

$$V = \frac{U.[E].[S]}{K+[S]},$$

where $[E]$ and $[S]$ represent the concentrations of enzyme and substrate, V the velocity of the catalysed reaction, U the maximum velocity for unit enzyme concentration, and K, the "Michaelis constant," the concentration of the substrate at which half the maximum velocity is attained. The reciprocal of K is the apparent affinity of the enzyme for the substrate.

This equation was found by Michaelis and Menten to hold with great accuracy for the action of saccharase on sucrose, and has since been found to fit the results obtained with many other enzymes. The full accounts of the many applications of this equation which are given by Kuhn [1925] and Haldane [1930] leave no doubt that the hypothesis of a stoichiometric enzyme-substrate compound is in good accord with the facts of enzyme kinetics.

The p_H*-activity curve.*

Many enzymes give p_H-activity curves showing an optimum p_H about which the curve is more or less symmetrical. Several authors have interpreted these curves as being due to the ionisation of the enzyme as an ampholyte, the isoelectric or un-ionised form of the enzyme alone being catalytically active, activity being determined not by the charge on the colloidal enzyme complex as a whole, but solely by the state of ionisation of one definite acidic and one basic group in the enzyme molecule. This view is supported by a considerable body of evidence, which is of two types: that based upon the shape of the curve, and that drawn from the effects of salts on the reaction rate.

Quantitative deductions from the shape of the curve can only be made when it covers eight or more p_H units. On the ampholyte theory, the curve would fall away from the optimum in a Henderson-Hasselbalch curve if the affinity did not change with p_H. If the affinity falls as the p_H recedes from the optimum, the curve will be steeper than a true dissociation curve, to an extent depending on the change in affinity with the p_H. These theoretical predictions have been realised, for instance by Josephson [1925] for β-glucosidase, and for saccharase, which is discussed by Kuhn [1925] and Haldane [1930]. Evidence from the effects of salts is given in such studies as those of Myrbäck [1926, 1] on saccharase and [1926, 2] amylase, and by Mann and Woolf [1930] on fumarase. These authors found that salts affected the velocity of the catalysed reaction in just the way that would be expected if the salt, or one of its ions, combined with the enzyme and altered the p_K value of one

or both of the critical ionisable groups, and hence the proportion of enzyme molecules in the isoelectric or active form at any given p_H.

Now there are many enzymes which do not give a p_H-activity curve of the symmetrical type, as for example the dehydrogenases [Dixon and Thurlow, 1924; Quastel and Whetham, 1924; and unpublished observations in this laboratory] and catalase [Michaelis and Pechstein, 1913]. Though it does not seem to have been pointed out before, the ampholyte type of curve is not characteristic of all enzymes, but only of those of a particular class—the enzymes which catalyse reactions in which water is one of the substrates. The non-hydrating enzymes mentioned above give a curve which rises in the acid range until a maximum velocity is reached, which persists unchanged until the enzyme begins to be destroyed by excessive alkalinity. Such a curve suggests that the enzyme acts as an ionised acid or an un-ionised base, and this suggestion is borne out by the fact that Michaelis and Pechstein [1913] found that the p_H-activity curve of liver catalase is a very accurate Henderson-Hasselbalch curve, which is shifted by salts in a manner analogous to the p_K-shifts found for the hydrating enzymes.

The enzyme a chemical compound.

All the kinetic results indicated above suggest strongly that an enzyme is a definite chemical structure. Further reasons for this conclusion are given by Willstätter [1927] in a summary of his work on enzyme purification, and by Haldane [1930] on several other grounds.

3. The addition compound theory.

The activation of the substrate.

It is obvious that the substrate must be distorted or activated in some way at the enzyme in order that reaction may occur, and various mechanisms, such as the electric field of Quastel [1926], have been proposed to account for this activation. All such theories are compelled to assume a double mechanism at the enzyme, the activating mechanism and some chemical grouping which will bind the substrate while it is being activated and undergoing reaction [*cf.* Quastel and Wooldridge, 1927, 1, 2]. It seems to the writer that this double mechanism is an unnecessary assumption. When the substrate is bound at the enzyme, becoming part of a temporary enzyme-substrate complex, the very chemical forces which bind it must surely produce sufficient distortion in its molecule to make it abnormally reactive. Until the chemical nature of enzymes is discovered, the nature of these forces can only be the subject of more or less plausible speculation. It seems likely, however, that since substrates are often stable, saturated molecules—*e.g.* succinic acid—the union with the enzyme is of the nature of a multiple co-ordination compound or chelate ring, which, as shown by Sidgwick [1927], involves the polarisation of the molecules which are so linked. But whether this or some other

mechanism is the true one, it seems to the writer an economy of hypotheses to assume that the substrate-binding and substrate-activating processes are one and the same until evidence to the contrary is produced. This view is also put forward in a slightly different form by Haldane [1930]. It may be assumed, then, that when the enzyme and substrate are combined, the chemical structures of the molecules are so altered that the complex will readily undergo further tautomeric changes, which constitute the actual catalytic process. The nature of these changes is discussed below.

The mode of action of a hydrating enzyme.

A convenient illustrative enzyme is fumarase [*cf.* Woolf, 1929], which catalyses the reversible reaction

$$HOOC.CH:CH.COOH + H_2O \rightleftharpoons l\text{-}HOOC.CH_2.CH(OH).COOH$$

since the complication is absent of the hydrated product splitting into two molecules, and the forward and reverse reactions can be readily induced, so that the mechanism of both can be discussed. The kinetic results already considered show that the conversion of fumaric to malic acid can only occur at an enzyme molecule which is combined with fumaric acid and in the isoelectric form. It may therefore be assumed that for reaction to be effected there must be present at the enzyme a fumaric acid molecule, a hydrogen ion, and a hydroxyl ion. Now the reaction product, malic acid, is formed by the addition of these three bodies. It is therefore a natural step to assume that the enzyme can only act in the fully hydrated form because the hydrogen and hydroxyl ions are substrates for the reaction, as well as the fumaric acid.

An enzyme molecule can be pictured uniting with a molecule of fumaric acid. In the majority of cases, the substrate molecule will dissociate off unchanged. But while the substrate is at the enzyme, its structure is distorted, it is polarised by the chemical forces holding it there, in such a way that it is able to "accept" a hydrogen and a hydroxyl ion at the two carbon atoms of the structure —CH:CH—. If these two ions are also present at the enzyme at the same time, in a given proportion of cases the enzyme-fumaric-water addition compound will tautomerise so that the water ions leave the enzyme and add themselves across the double bond of the substrate, which will thus become free to dissociate off as malic acid. When this occurs, it is important to notice that the enzyme is left with neither a hydrogen nor a hydroxyl ion, *i.e.* in the zwitterion form.

There are thus four forms of the enzyme to consider—the dissociated acid, the dissociated base, the fully hydrated, and the zwitterion form. The last two together constitute the isoelectric form of the enzyme, which will be partitioned into hydrated and zwitterion forms in a constant ratio, which is independent of the p_H.

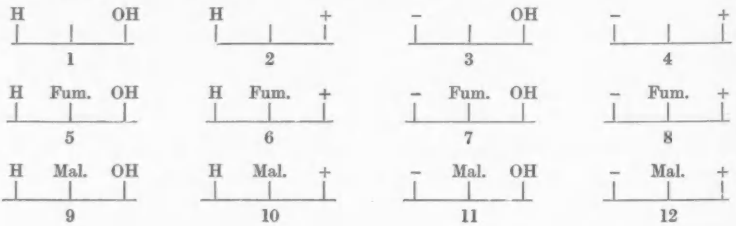
Consider now the reverse reaction. When a molecule of malic acid combines at the enzyme, it too will undergo polarisation so that the elements of

water become dissociable as ions. If the enzyme already has a hydrogen or a hydroxyl ion, it will be unable to take up water from the substrate, and reaction cannot occur. If however it is in the zwitterion form, in a proportion of cases water will leave the substrate and combine with the enzyme, the substrate then dissociating off as fumaric acid and leaving the enzyme fully hydrated. The equilibrium can therefore be represented

$$[E^{H}_{OH}] + \text{Fumaric} \rightleftharpoons [E^{H}_{OH}].\text{Fumaric} \rightleftharpoons [E^{-}_{+}].\text{Malic} \rightleftharpoons [E^{-}_{+}] + \text{Malic}.$$

The mechanism here suggested thus conforms to the thermodynamic requirement that the reverse reaction in a reversible process shall pass through exactly the same stages, in the opposite order, as the forward process. The essential features of the process may be expressed as follows. The fumaroid skeleton, when combined with the enzyme, is so polarised that it acquires temporary acidic properties at one carbon atom and basic properties at the other. The complex thus becomes a dibasic acid and diacidic base, and the hydrogen and hydroxyl ions will partition themselves, statistically, between the acidic and basic groups of the enzyme and the substrate in a proportion determined by the relative affinity of the enzyme and the substrate for the water. It is obvious that from such a process a true thermodynamic equilibrium will be obtained.

In a system containing fumarase, fumaric acid and water there are the following forms of the enzyme to be considered:



Form 5 is catalytically active in the forward reaction and form 12 in the reverse direction. All these forms are in equilibrium with one another and with fumaric acid, malic acid and the ions of water in the medium, and the affinity of each ionic species of the enzyme for fumaric acid and malic acid will, in general, not be the same. The state of affairs is therefore rather complex. It is however amenable to treatment by the law of mass action, and is then found to lead quantitatively to the kinetic results which are actually found by experiment, and which are set out in Section 2 of this paper.

The above line of argument applies to any hydrating enzyme, with the complication that when the catalysed reaction is a hydrolysis, the reverse reaction involves two substrates, each of which must combine with the enzyme, with its own characteristic affinity.

The mode of action of a dehydrogenase.

Succinoxidase will be taken as an example, since it is the only dehydrogenase whose thermodynamic reversibility has been proved experimentally, and methylene blue will be considered to be the hydrogen acceptor. The catalysed reaction then consists of the transfer of two hydrogen atoms from the donator to the acceptor. Many considerations render it probable, however, that the actual act of oxidation consists in the transfer of two electrons to the dye from the donator, which has previously lost two hydrogen ions, the process being completed by the acquisition of two hydrogen ions by the charged acceptor. One must therefore visualise the donator being polarised by its combination with the enzyme so as to acquire acidic properties at the structure $—CH_2.CH_2—$, the enzyme, in a given proportion of cases, taking up the two temporarily ionisable hydrogens from the donator in the same way as fumarase takes up the temporarily ionisable hydrogen and hydroxyl from malic acid. In order to be able to do this, the enzyme must itself be an ionised acid, which is precisely the conclusion that is indicated by the shape of the p_H-activity curve.

There is good kinetic evidence that the donator must combine with the enzyme. Reasons were given by Woolf [1929] for supposing that the acceptor must also combine. These reasons are reinforced by the work of Bertho [1929], who found that quinone, acting as hydrogen acceptor with the dehydrogenases of certain lactic acid bacteria, gave a good Michaelis curve. Unpublished observations by the writer show that the same is true for methylene blue and other dyes acting as hydrogen acceptors with a variety of dehydrogenases. In order to be catalytically active, therefore, a dehydrogenase molecule must have combined at it a molecule of donator and a molecule of acceptor, and be itself dissociated as an acid. If DH_2 and D represent the reduced and oxidised forms of the donator, AH_2 and A the corresponding forms of the acceptor, E the enzyme and * an electron, the sequence of changes may then be formulated as follows:

$$[E^{**}]+DH_2+A \rightleftharpoons [E^{**}]_A^{DH_2} \rightleftharpoons [EH_2]_A^{D^{**}} \rightleftharpoons [EH_2]_{A^{**}}^{D} \rightleftharpoons [E^{**}]_{AH_2}^{D} \rightleftharpoons [E^{**}]+D+AH_2.$$

In this scheme the enzyme is represented as a dibasic acid, but the same general result would be obtained if it were taken as monobasic, though the formulation would be a little more lengthy. The doubly charged enzyme combines with both substrates. The donator is activated, and parts with two hydrogen ions to the enzyme. The two excess electrons are then transferred from the donator to the acceptor, this being the actual act of oxidation. The acceptor then acquires two hydrogen ions, and both substrates dissociate off in the altered condition, leaving the enzyme in its initial state. Since all the intermediate forms are in dynamic equilibrium, it is probable that only a small proportion of the enzyme-substrate complexes formed will be completely

transformed, and most will dissociate into enzyme and unchanged substrates. When the sequence of changes is read in the opposite order, it represents the reverse reaction—*e.g.* the reduction of fumaric to succinic acid by leucomethylene blue. This mechanism, like that suggested for the hydrating enzymes, is therefore thermodynamically sound.

Similar considerations could be applied to other types of enzyme action, but these two examples suffice to indicate the scope and method of the theory. While it is claimed that the central idea is new, the writer wishes to acknowledge that he has freely drawn on the hypotheses of many previous workers, and adopted from them whatever elements seemed to him of value. It is claimed for this theory that it contradicts no known facts; that it gives a unitary explanation of the phenomena of enzyme kinetics, including p_H-activity curves, which no previous theory has professed to explain; that the mechanism of enzyme action proposed is a plausible one; and that it is a useful working hypothesis, in that it enables many quantitative predictions to be made, which can be tested by clear-cut experiments. It has certainly proved of extraordinary value as a working guide to the writer, and it is hoped to publish in the near future several enzyme studies suggested by the theory. The writer hopes that it may also prove of value to other workers in the field of enzyme action.

SUMMARY.

The theory is proposed that an enzyme is a definite chemical compound, which is able to form an addition compound with all its substrates, the actual process of catalysis consisting of a series of tautomeric changes in this enzyme-substrate complex, as a result of which, in a certain proportion of cases, it is able to dissociate into free enzyme and reaction products. This theory is shown to account in a quantitative manner for the observed effects of substrate concentration, p_H, and salts on the velocity of the catalysed reaction.

It is a pleasure to express my gratitude to Prof. J. B. S. Haldane for his constant and illuminating criticism, and to Sir F. G. Hopkins for his interest and encouragement.

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XLIII. ON THE INADEQUACY OF WHITE WHEAT FLOUR AND FISH MEAL FOR PROLONGED GROWTH OF PIGS AND ITS AMELIORATION WITH YEAST OR STOUT.

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(*Received December 10th, 1930.*)

Introduction.

Plimmer, Rosedale and Raymond [1927] used a diet of white flour and fish meal with varying quantities of marmite and dried yeast in maintenance experiments on pigeons and chickens. They concluded that the calories in the diet need balancing by vitamin B ($B_1 + B_2$).

The quantity of dried yeast required was found, in the case of chickens, to be 6 to 10 % in a food containing no vitamin B.

Very little work however has been carried out on the requirements of pigs for the yeast vitamins. Gibson and Concepcion [1916] in their experiments on pigs fed exclusively on cow's milk from the age of 17 days, obtained evidence of neuritis by the histological examination of the sciatic and pneumogastric nerves in pigs killed 34 days and 60 days after the start of their experiments, but the large daily intake of a pig has prevented the use of these animals for vitamin B investigations.

The experiments reported below were undertaken to answer the following questions.

(1) If a diet of white wheat flour and fish meal was inadequate for the growth of pigs.

(2) If dried yeast as a recognised and available source of B vitamins would improve the diet.

(3) If a stout which contained some living yeast (see p. 357) had the property of ameliorating a diet deficient in B vitamins.

The pig is able to consume a sufficient quantity of stout and thus the difficulties of previous concentration or extraction, necessary in the case of pigeons and rats, are avoided.

Experiments have, nevertheless, also been carried out with rats, and these will form the subject of another communication.

Experimental.

Five experiments are decribed below, involving the use of 59 pigs. In all these experiments the animals were divided into comparable groups, all receiving a basal diet of white wheat flour and white fish meal.

The flour was specially ground from English wheat by a local miller and was free from germ and husk.

The chemical composition of the constituents of the basal ration was as follows:

	Wheat flour %	White fish meal %
Moisture	19·85	10·32
Oil	2·98	6·23
Proteins	7·90	63·10
Starch, *etc.*	68·82	—
Ash	0·45	20·35
	100·00	100·00

A mixture of 90 % flour and 10 % fish meal showed a starch equivalent of 69·05 and an albuminoid ratio 1:5·4; the digestible proteins were 10·94 %. Such a mixture would not from its composition seem unsuitable for pigs and when the pigs are nearing bacon weight the fish meal could be cut down to 5 % of the mixture, which was done in two of the experiments reported below; the albuminoid ratio was then widened to 1:7·30 and the digestible protein reduced to 8·39 %. Although the fish meal might be assumed to supply a sufficiency of the vitamins A and D, cod-liver oil was also given at the commencement of each experiment. The ash in the fish meal was considered ample for mineral requirements.

The pigs in the positive control group received, in addition, dried brewer's yeast at the rate of 2 ounces per pig per day, except in Exp. 4 (see below).

The dried yeast had the following composition:

Moisture	Oil	Proteins	Carbohydrates	Woody fibre	Ash
9·58 %	0·46 %	45·91 %	32·13 %	3·15 %	8·77 %

The stout was fed in varying quantities as given below. It was in good condition and a fresh supply was received in bottles weekly.

The food for each lot of pigs was weighed out every morning and mixed with water in a large covered earthenware jar.

With the exception of Exps. 3 and 4 the dried yeast was mixed with the food in the morning. In these experiments the yeast was mixed at the time of feeding.

The stout was always mixed with the food at feeding time. The pigs were fed at 8 a.m. and 4 p.m. each day. They were weighed every Tuesday at noon.

A short detailed account of the experiments is given below. Although these differ in minor details the first three may be regarded as repeat experiments, and the results in general are confirmed by Exps. 4 and 5 as will be seen by reference to the charts, Figs. 1 and 2.

In Exp. 4 the negative control group repeated previous experiments but

the other two groups only differed from them in that half an ounce daily of dried brewer's yeast was given per pig in one lot and the equivalent of ½ ounce per pig as a water extract daily in the other group.

In Exp. 5 the negative control group repeated previous experiments, but the three other lots differed from them in that the mixture of flour and fish meal was cooked by blowing steam into the pails in which it had been weighed out.

Exp. 1. After a preliminary period of 1 week to accustom the animals to the diet the experiment was started on January 22nd, 1929. A selection was made from two litters, 8 Large White pigs 73 days old and 4 Large White × Middle White pigs 87 days old being chosen.

10 cc. of cod-liver oil were given to each pig daily for the first 55 days of the experiment. The ration was altered to 95 % flour and 5 % fish meal on the 74th day.

The bedding of the animals was changed on April 9th from cereal straw to wood shavings, as it was thought that they were eating enough straw to make a possible difference to the results. This change apparently did not make any difference.

The group shown at the bottom of Fig. 1 received half a pint of stout per pig per day until the 80th day when the quantity was increased to 1 pint.

Exp. 2. This experiment was started on April 30th, 1929. Ten Large White × Middle White pigs all from one litter were available. They were 79 days old at the start of the experiment. The pigs were divided into groups of 4, 3 and 3.

Cod-liver oil was given to all the pigs until the 39th day at the rate of 10 cc. per pig per day. On the 51st day the basal diet was altered to 95 % wheat flour and 5 % fish meal. Wood shavings were used as bedding throughout the experiment.

The pigs in the third group received 1 pint of stout per day.

Exp. 3. This experiment was started on February 18th, 1930. Purchased pigs of unknown origin were used. The pigs were divided into 4 groups of 3 pigs each. Two groups were kept as controls, one received stout and one received 2 ounces of dried brewer's yeast.

Cod-liver oil was given to all the pigs from February 26th to March 26th at the rate of 10 cc. per pig per day, after which date only the pigs in one of the negative control groups (group No. 1) continued to receive the oil.

The pigs did not have any bedding in this experiment.

Exp. 4. This experiment was started on June 25th, 1930. Eleven Middle White × Large White pigs were available; they were farrowed on the Institute's farm on April 14th, 1930.

They were divided into three pens as follows:

No. 1. 1 sow and 2 hogs fed on 90 % flour 10 % fish meal.

No. 2. 1 sow and 3 hogs fed on 90 % flour 10 % fish meal + ½ oz. yeast.

No. 3. 1 sow and 3 hogs fed on 90 % flour 10 % fish meal + yeast extract.

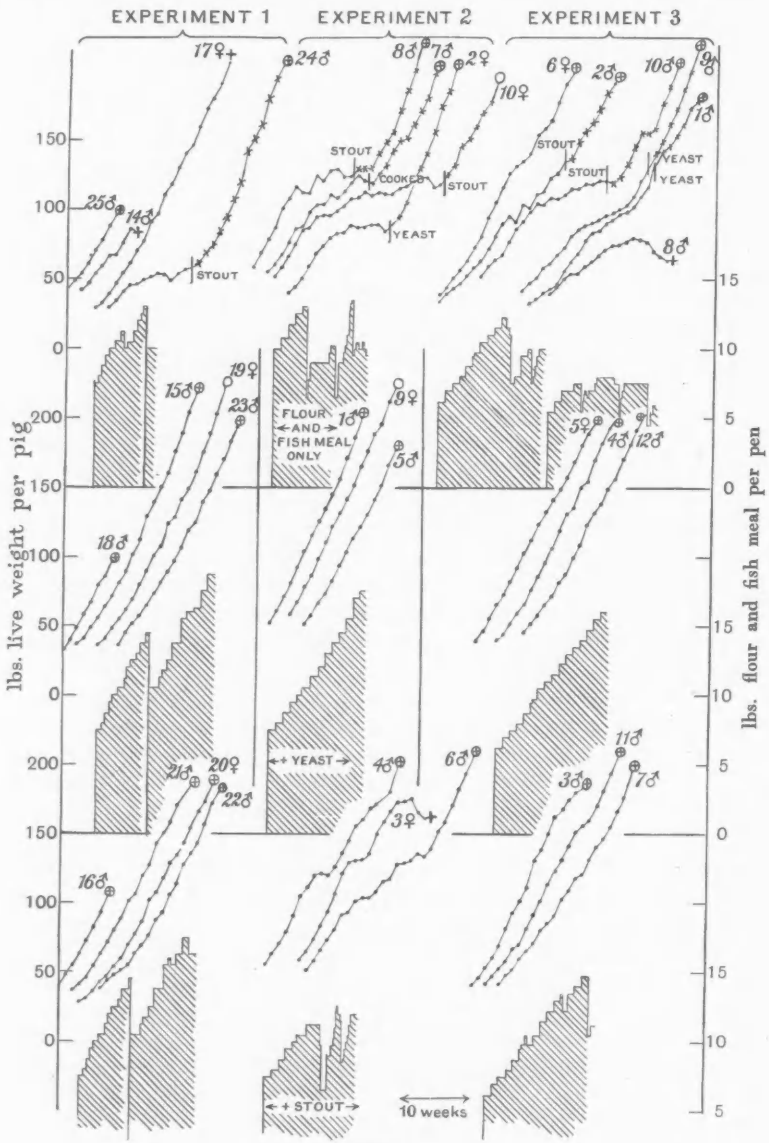

Fig. 1.

Figs. 1 and 2. Results of Exps. 1 to 5 are shown in vertical columns. Growth curves of individual pigs in lb. live weight are arranged above the daily intake of flour and fish meal per pen. The growth curves and intake records of each experiment started on the same day but have been spaced to avoid overlapping. The terminal signs at the end of each growth curve indicate ⊕ = Pigs sent to butcher. + = Pig died. ○ = Pig removed from experiment.

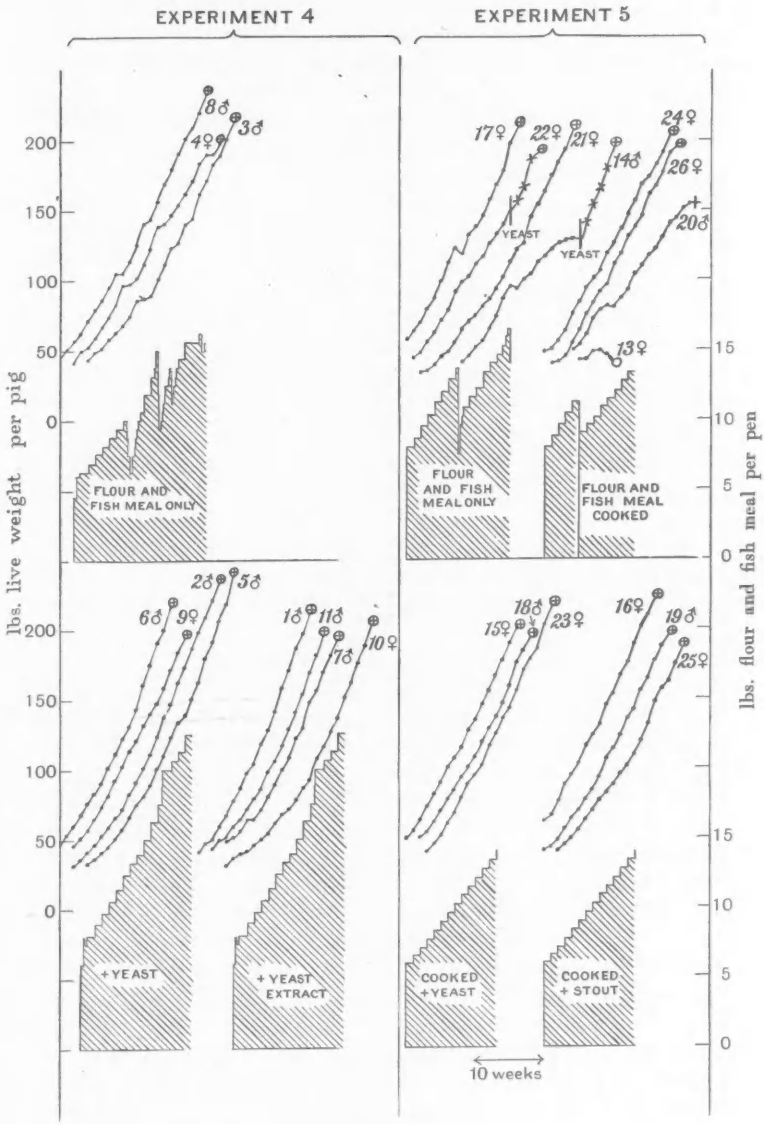

Fig. 2.

Curative treatment of pigs in negative control groups started at the vertical line, the nature of the treatment being indicated.

The top sets of growth curves and intake diagrams in both figures are negative controls, flour and fish meal only.

In the lower sets yeast and stout were added as shown.

The yeast extract was made by pouring boiling water on dried brewer's yeast; after stirring for 1 minute the infusion was left to cool and decanted 1 hour later through a cheese cloth. The liquid was squeezed out and after standing for a day was again decanted from sediment.

The total solids in the extract averaged 2·75 %; 74 cc. of this extract derived from 7·4 g. (= 0·26 oz. and containing 2·03 g. dry extracted matter) were fed to each pig daily. The yeast extract required to maintain the growth of a rat on a diet free from vitamin B was 0·5 cc. per rat daily. No bedding was given to the pigs in this experiment.

The yeast extract was not taken readily at first and had to be increased little by little to an amount equivalent to one quarter of an ounce of the original yeast per pig per day.

Exp. 5. This experiment was started on March 4th, 1930. Fourteen purchased pigs of unknown origin and mixed breed were used; they were similar to those used in Exp. 3. They were divided into 4 lots as follows:

No. 1. 3 sows and 1 hog fed on 90 % flour and 10 % fish meal uncooked.

No. 2. 3 sows and 1 hog fed on 90 % flour and 10 % fish meal cooked.

No. 3. 2 sows and 1 hog fed on 90 % flour and 10 % fish meal cooked + 2 oz. yeast.

No. 4. 2 sows and 1 hog fed on 90 % flour and 10 % fish meal cooked + stout.

As in Exp. 3 the pigs had no other bedding than boards in their sleeping pens.

Results.

The growth curves of the pigs are set out in Figs. 1 and 2 with the intake of each group. The comparable groups in each experiment are arranged in vertical columns.

When Exp. 1 had been in progress 48 days there was little to choose between the groups and it was thought that negative results only might be obtained. One pig from each group was, therefore, sent to the butcher as a "porker." The food intake of the pen was reduced at this date in proportion to the weight of the pig removed, this is indicated by a break in the intake graph.

In all the experiments the differences between the weights of the individual pigs in the three groups were not very marked at the end of the first 40 to 50 days on the diet. The exceptions were pig No. 24 in Exp. 1 which did not thrive on the flour and fish meal diet and pig No. 13 in Exp. 5 which did not grow on the cooked flour and fish meal and had to be taken out of the experiment as it was feared it was developing an illness.

If we had sent the pigs to the butcher at porker weight the results would have been indefinite. By keeping the pigs on to bacon weight of 200 lb. it becomes quite clear that there are marked differences in the development of the three groups.

In every group receiving 2 oz. of yeast the growth was very uniform and the pigs seemed in good health. The pigs receiving stout exhibited greater differences between individual members of the same group, but were also healthy and free from sickness (except sow No. 3, Exp. 2, which died from generalised peritonitis). They were, however, much more uniform in growth and better in health and appearance than the negative controls.

The negative control groups showed features which have not infrequently been seen in experiments of this character, namely that whereas some of the pigs grew satisfactorily others suffered from lack of appetite, sickness, and in some cases death.

Reference to the charts shows that the growth was irregular, some pigs doing better than others. The growth alone does not give an adequate picture of the unthrifty rough-coated appearance of these negative control pigs.

It is necessary to discuss these animals in greater detail and to give some particulars of the *post mortem* findings.

There were 11 sows and 14 hogs fed on a diet of flour and fish meal only. Of these 3 hogs and 1 sow died, 8 hogs and 3 sows were cured (see below) 1 sow was removed from the experiment and 3 hogs and 6 sows survived without treatment.

All the pigs which were sent to the butcher (marked ⊕ on charts) and all pigs which died (marked + on charts) were submitted to *post mortem* examinations and in some cases bacteriological and histological examinations were also made.

In this work we are greatly indebted to Mr W. L. Little for *post mortem* examinations, and Dr J. Mills (Pathologist to the Berkshire Hospital) for histological examinations.

In this preliminary note it is not proposed to go into details but Dr Mills reports that "there was no morbid anatomical appearance sufficiently typical of vitamin B deficiency disease to enable one to state definitely from the *post mortem* examination that death was due to such deficiency.

In some of the *post mortem* examinations the sciatic nerves on histological examination showed extravasation of blood into the connective tissue between the bundles of nerve fibres but it is doubtful whether this was due to disease or an artefact produced in removing the nerves, in any case the nerve fibres themselves appeared normal."

Curative treatment.

After varying periods on the basal diet, stout or dried yeast was given to a number of the negative control pigs which failed to grow. This caused a marked increase in the rate of growth in all cases, as can be seen in Table I.

In Exp. 2, hog No. 7 was given cooked food when, after 102 days on uncooked food, its weight began to decline as is shown in Table I and Fig. 1. The rate of growth of this pig improved from the time of the change of diet. In Exp. 5, however, where cooked food was given from the commencement

Table I. *Curative treatment.*

Exp. No.	Pig No.	Sex	Wt when curative treatment of negative controls commenced	6 weeks before treatment: Diet	6 weeks before treatment: lbs. live wt gain per day	6 weeks following treatment: Diet	6 weeks following treatment: lbs. live wt gain per day
				Flour and fish meal daily		Flour and fish meal daily	
1	24	Hog	58·5	Only	0·167	+1½ pints stout	1·155
	23	„	57	+2 oz. yeast	**0·952***	+2 oz. yeast	**1·155**
2	2	Sow	84	Only	−0·012	+2 oz. yeast	1·333
	7	Hog	121·5	„	0·417	Cooked	0·690
	8	„	124	„	0·286	+1½ pints stout	0·774
	10	Sow	118·5	„	0·060	+1½ pints stout	1·202
	1	Hog	**126**	+2 oz. yeast	**1·548**	+2 oz. yeast	**1·762**
	9	Sow	**121·5**	+2 oz. yeast	**1·500**	+2 oz. yeast	**1·583**
3	1	Hog	132	Only	0·917	+2 oz. yeast	1·095
	2	„	135	„	0·738	+2½ pints stout	1·190
	9	„	130	„	0·798	+2 oz. yeast	1·667
	10	„	122	„	0·274	+2½ pints stout	0·786
	4	„	**130**	+2 oz. yeast	**1·524**	+2 oz. yeast	**1·500**
5	14	Hog	129	Only	0·381	+2 oz. yeast	1·857*
	22	Sow	151	„	1·167	+2 oz. yeast	1·429*
	18	Hog	**128**	Cooked +2 oz. yeast	**1·190**	Cooked +2 oz. yeast	**1·560**
	15	Sow	**146**	Cooked +2 oz. yeast	**1·333**	Cooked +2 oz. yeast	**1·548***

* Calculated on a shorter period than 6 weeks.

The figures in heavy type are given for comparison. They represent pigs from the same litter in each experiment fed with an addendum of 2 oz. of yeast from the start. They are taken from the weighing at which they most nearly corresponded to pigs in the negative control groups.

of the experiment there appeared to be no marked improvement in favour of the cooked food when compared with a control group of pigs receiving uncooked food.

Discussion.

All five experiments indicated that the basal diet was inadequate for the nutrition of pigs for a prolonged period.

At the start and for periods of about 7 weeks the pigs on the basal diet grew well and consumed almost as much food as the pigs in the other lots. In the earlier part of the experiment 2 oz. extra food were offered to balance the yeast given to other pigs.

It was frequently recorded that, even when the negative control pigs received less food than those in the other two pens, they did not clear up their allowance. When this happened the quantity was reduced, for we have always found that if pigs are offered more than they require their appetite goes off. They were, however, always tempted with more food as soon as they had cleared up the quantity given. In Exp. 4 the food refused was dried and weighed. The regularity and uniformity of the growth and food consumption in the lots receiving yeast are most striking when compared with the negative controls. The pigs receiving stout come between the two groups. The pigs on stout received less dry matter in the supplement than the yeast-fed pigs.

The quantity of yeast they could have received in the stout was about 1/1000th that supplied by 2 ozs. of dried yeast.

Summary.

A basal diet of wheat flour and fish meal has proved inadequate for the sustained growth of pigs.

Additions of 2 oz. per pig daily of dried brewer's yeast made the diet quite satisfactory. In Exp. 4, ½ oz., or even extract equal to ¼ oz., of yeast made the diet satisfactory. Stout given in quantities varying from ½ pint to 2 pints per pig daily had a similar but less marked effect.

We wish to express our indebtedness to Lord Iveagh for a grant which has made it possible to undertake work on this and other problems of nutrition, and to Messrs Arthur Guinness, Son and Co. for supplying the stout and dried yeast.

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XLIV. THE FAT-SOLUBLE VITAMIN REQUIREMENTS OF THE CHICK.

I. THE VITAMIN A AND VITAMIN D CONTENT OF FISH MEAL AND MEAT MEAL.

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(*Received December 4th, 1930.*)

In a recent communication [McFarlane, Graham and Hall, 1930] it was shown that normal chicks could not be grown in confinement when fed synthetic diets which supplied what are believed to be adequate amounts of the vitamins known to be essential for growth in the chick. The birds during the 4th to the 6th week of growth developed certain deformities and paralysis of the limbs, a condition commonly designated as leg-weakness, the cause of which could not be adequately explained by what is now known of the nutritional requirements of the chick. Much difference of opinion exists in the literature in regard to the cause of this pathological condition, which has long been the greatest difficulty in rearing chicks in the laboratory on synthetic diets. It would appear either that more than one dietary factor may be responsible for this condition, or that there is more than one form of leg-weakness. One type of leg-weakness studied by many workers, notably Hughes and Titus [1926]; Mitchell, Kennard and Card [1923]; Hart, Halpin and Steenbock [1922]; Emmett and Peacock [1923] and more recently by Mussehl and Ackerson [1930] and by Massengale and Nussmeier [1930], can be prevented by the addition of cod-liver oil to the diet. As to whether this effect is due to vitamin A or vitamin D is still disputed. The preponderance of evidence indicates that it is a vitamin A deficiency which is the cause of this particular type of leg-weakness and that this leg deformity is not a rachitic condition [see Pappenheimer and Dunn, 1925]. All these investigators agree that the fat-soluble vitamin requirements of the chick are high and that to prevent the incidence of this form of leg-weakness, in chicks fed in the laboratory, at least 2 % of a high grade cod-liver oil must be added to the diet.

Plimmer, Rosedale and Raymond [1927] believe they have confirmed the findings of Dunn [1924] that as little as 0·5 % of cod-liver oil of good quality is sufficient to rear chicks to maturity. They obtained little or no growth and lost all the chicks within 4 weeks on feeding a diet composed of white rice

78 %, dried yeast 17 %, and fish meal 4 %. The addition of 0·4 or 0·6 parts of cod-liver oil (their Group XXXII A), did not materially improve the results. On changing the diet to white rice 75·3 %, dried yeast 16 %, fish meal 8 %, and cod-liver oil 0·5 % (their Group XLIII), excellent growth was obtained. They themselves express surprise that "birds could be reared on such small quantities of cod-liver oil as 0·1 and 0·2 %." Again with a diet composed of maize 89 %, fish meal 10 %, and cod-liver oil 1 %, normal rearing was obtained, but when the amount of cod-liver oil was reduced to 0·25 % or no cod-liver oil was given, rickets developed. The chicks, however, appear to have grown fairly well and only one chick was lost when the amount of cod-liver oil was as little as 0·25 %. From these results they have concluded that as little as 0·5 % of cod-liver oil of good quality is sufficient to rear chicks to maturity. Their conclusion is evidently based on the assumption that the fish meal contained no fat-soluble vitamins or possibly the fish meal had been freed from fat by extraction with ether, but this is not mentioned in their paper. The only essential difference between the diet fed to Group XXXII A which was a failure and that fed to Group XLIII which grew exceptionally well was in the fact that twice the quantity of fish meal was fed to the latter group; this improvement, we presume, they attributed entirely to the increase in the percentage protein in the diets. Again with the diet containing large quantities of maize, the failure of 10 % fish meal to protect the birds against rickets when no cod-liver oil was added may not indicate that fish meal contains little or no vitamin D but rather that maize compared with white rice, as shown by Mellanby [1929], contains a large amount of the anticalcifying factor which interferes with the deposition of calcium salts.

That fish meal may contain a very considerable amount of the fat-soluble vitamins while meat meal is not so endowed was suggested to us by the experiments of Graham and Smith [1929]. In a study of the influence of various protein materials on reproduction in the chick these workers found that the addition of 2 % of cod-liver oil to a diet for laying hens, containing 10 % of meat meal, had a pronounced effect in increasing the hatchability of the fertile eggs. The same basal diet but with 7·5 % of white fish meal as the protein supplement and without cod-liver oil gave just as high a percentage hatchability of the fertile eggs as that obtained with the meat meal diet containing 2 % cod-liver oil. The addition of 2 % cod-liver oil to the fish meal diet resulted in little or no improvement in the hatchability of the eggs.

The experiments to be described were undertaken to determine whether the vitamin A and vitamin D content of the fat of fish meal was different from that of the fat of meat meal. The results show conclusively that there is a difference, *i.e.* the particular white fish meal used in these experiments contained a very considerable amount of both vitamin A and vitamin D. In view of this the results of Plimmer *et al.* [1927] might legitimately be interpreted as showing that the vitamin A or vitamin D requirements of the chick are high and that the amount of vitamin A and vitamin D in the quantity of

fish meal fed in their experiments, while quite considerable, was not sufficient to protect the birds against rickets or a vitamin A deficiency, but that complete protection and normal growth were secured by adding 0·5 % cod-liver oil. Their findings, which constitute the most recent attempt recorded in the literature to assay the fat-soluble vitamin requirements of the growing chick, cannot, in the light of the results reported below, be considered reliable unless they have taken the precaution to remove by ether extraction the vitamin A and vitamin D which the fish meal used in their basal diet undoubtedly contains. Since this is not recorded in their paper, it can only be assumed that the fish meal added to their diet a considerable amount of the fat-soluble vitamins and that the vitamin A and vitamin D requirements of the chick may be relatively high instead of, as they have concluded, remarkably low.

Experimental.

The fish meal used in these experiments was a white fish meal prepared from non-oily fish such as cod (from which the liver had been removed) hake, plaice, haddock, skate and ling, etc. The meat meal was a finely ground meaty substance consisting of the residues of animal tissues but exclusive of hoof and horn and from which the fat had been partially extracted by hydraulic pressure after the direct drying of the material in revolving drums under 40 lbs. steam pressure (240°). The content of ether-soluble substances in fish meal is relatively low, while that in meat meal is much greater (see Table I). The cod-liver oil used was a high grade medicinal oil obtained from W. A. Munn, St John's, Newfoundland.

Table I. *Percentage composition of fish meal and meat meal.*

	Fish meal	Meat meal
Moisture	5·90	3·84
Crude protein (N × 6·25)	75·77	55·90
Ether extract	3·86	14·58
Crude fibre	0·23	1·22
Total ash	17·63	19·51
Silica	1·90	0·44
P_2O_5	5·55	6·85
CaO	5·97	8·55
MgO	0·88	0·59
Na_2O	1·81	1·02
K_2O	0·48	0·44
Fe_2O_3	0·016	0·04
Cu (mg. per kg.)	4·20	5·40
Ratio calcium : phosphorus	1·6 : 1	1·9 : 1

To ascertain whether fish meal or meat meal contains considerable amounts of vitamin A or vitamin D, the rates of growth of nine groups of barred rock chicks from 1 day to 8 weeks of age on the following diets were determined.

Fish meal groups.

Group I. Fish meal 14·8 %, marmite 15 %, white rice 70·2 %.

Group II. Same diet but with 2 % cod-liver oil replacing an equal amount of white rice.

Group III. Same diet as Group I, but with the chicks irradiated for 15 minutes daily from a quartz mercury vapour lamp at a height of 24 inches.

Group IV. Fish meal (freed from fat by extraction with ether) 14·6 %, marmite 15·0 %, white rice 67·4 %, and cod-liver oil 3 %.

Meat meal groups.

Group V. Meat meal 16·6 %, marmite 15 %, white rice 68·4 %.

Group VI. Same diet as group V but with 2 % cod-liver oil replacing an equal amount of white rice.

Group VII. Same diet as group V but with the chicks receiving ultra-violet irradiation for 15 minutes daily.

Group VIII. Meat meal (freed from fat by extraction with ether) 14·8 %, marmite 15·0 %, white rice 67·4 %, and cod-liver oil 3 %.

Control group.

Group IX. Caseinogen 12·2 %, marmite 15 %, white rice 65·8 %, cod-liver oil 3 %, and salt mixture 4 % [see Hart *et al.*, 1920].

The total protein (N × 6·25) of all these diets was approximately 18·5 %, being made up of 5·8 % from marmite, 2·6 % from white rice, and 10 % from each of the protein sources. The total ash of all the diets was approximately 4·4 %. The experiment was commenced with day-old chicks, 35 in each group, housed and managed in the laboratory under the same conditions as described by McFarlane, Graham and Hall [1930]. At the end of the 1st week the numbers were reduced to 30 chicks in each group, discarding those either showing evidence of constitutional weakness, or varying most widely from the mean weight of the group.

In Fig. 1 and Fig. 2 is shown the average growth in g. of the chicks receiving the different fish meal and meat meal diets. The weekly mortality in each group is also recorded on each growth curve in arabic figures, each value representing the number of chicks which died during the previous week. The growth of the control Group IX receiving caseinogen as the source of protein is recorded in each figure for comparison. No chicks were lost in this group during the first 8 weeks of growth.

We have also determined the calcium and inorganic phosphorus content of the blood-serum of a number of chicks in each group with the exception of Groups VII and VIII. In Fig. 3 and Fig. 4 are plotted the results of these determinations. The analyses at the end of the 1st week were made on the chicks which were being discarded from the experiment, it being found necessary to combine the blood from two chicks on the same diet to obtain sufficient blood for a determination. It was necessary to sacrifice two or three chicks in each group each week until the 8th, when sufficient blood for the analysis could be obtained from the brachial artery without the loss of the chick. Calcium was determined on 2 cc. of serum by the Clark-Collip modification [1925] of the Kramer-Tisdall method [1921]; inorganic phosphorus was determined on the supernatant fluid from the serum-calcium by the

Gunther-Greenberg modification [1929] of the Fiske and Subarrow procedure [1925]. For comparison we have also recorded the results of calcium and inorganic phosphorus determinations on the serum of chicks in the control caseinogen Group IX, of chicks receiving a rachitic diet and housed behind window-glass (Group X), and of chicks receiving the same diet as Group X but with 2 % of cod-liver oil added and housed under vita-glass (Group XI). The diet on which the chicks developed rickets was composed of yellow corn 37 %, ground wheat 50 %, oatmeal 25 %, buttermilk powder 10 %, meat

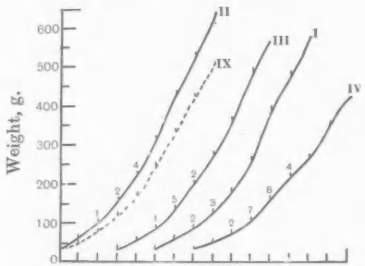

Fig. 1.

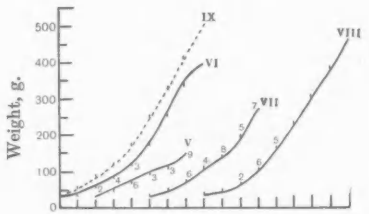

Fig. 2.

Fig. 1. The effect on growth of various additions to a diet composed of fish meal 14·8 %, marmite 15 %, and white rice to 100.

Curve I. No cod-liver oil added or ultra-violet irradiation.
" II. 1 % cod-liver oil added.
" III. Chicks received ultra-violet irradiation 15 min. daily.
" IV. Ether-extracted fish meal and 3 % cod-liver oil.
" IX. Caseinogen control diet and 3 % cod-liver oil.

NOTE. The arabic figures on each group curve in Figs. 1 and 2 give the number of chicks which died during the preceding week.

Fig. 2. The effect on growth of various additions to a diet composed of meat meal 16·6 %, marmite 15 %, and white rice to 100.

Curve V. No cod-liver oil added or ultra-violet irradiation.
" VI. 1 % cod-liver oil added.
" VII. Chicks received ultra-violet irradiation 15 min. daily.
" VIII. Ether-extracted meat meal and 3 % cod-liver oil.
" IX. Control caseinogen diet and 3 % cod-liver oil.

meal 12·5 %, alfalfa meal 3·5 %, bone meal 2·5 %, oyster shell 1·5 %, and salt 0·5 %.

DISCUSSION.

During the first eight weeks of growth the inorganic phosphorus content of the blood-serum of normal chicks (Groups IX and XI, Fig. 4) is fairly constant but with chicks receiving a rachitic diet (Group X) the inorganic phosphorus content of the serum drops to a very low level during the 3rd and 4th weeks, when the birds show a typical rachitic condition, and then slowly recovers to the original level. These changes in the inorganic phosphorus of the blood-serum of rachitic chickens are similar to those accompanying uncomplicated mammalian rickets.

It will be seen from Fig. 1 that good growth and very little mortality was obtained with a diet containing fish meal as the only source of the fat-soluble vitamins (Group I) and that the result was practically unchanged when chicks on the same diet received ultra-violet irradiation (Group II). The growth was,

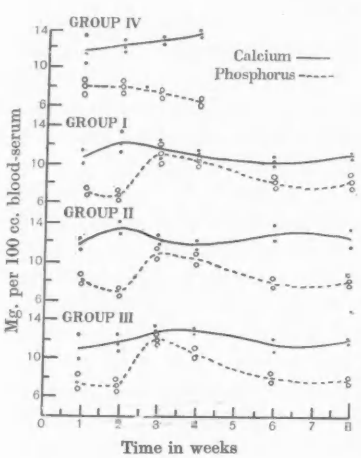

Fig. 3.

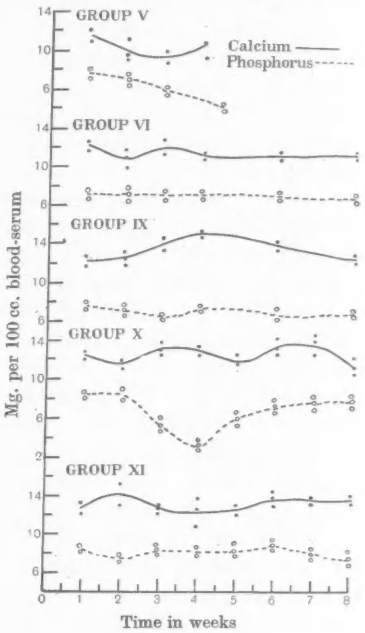

Fig. 4.

Fig. 3. Showing the effect on the calcium and inorganic phosphorus content of the blood-serum of chicks fed different fish meal diets.

Group I. No cod-liver oil. Group II. 1 % cod-liver oil.
„ III. Ultra-violet irradiation 15 min. daily.
„ IV. Fish meal ether-extracted and 3 % cod-liver oil.

Fig. 4. The effect on the calcium and inorganic phosphorus content of the blood-serum of chicks fed different meat meal diets.

Group V. No cod-liver oil. Group VI. 1 % cod-liver oil.
„ IX. Caseinogen control diet, 3 % cod-liver oil.
„ X. Rachitic diet, no cod-liver oil.
„ XI. Same diet as Group X but with 2 % cod-liver oil.

however, improved by adding 1 % cod-liver oil to the diet and was better than that attained by the caseinogen control Group IX. Fish meal which had been extracted with ether (Group IV) gave much the poorest growth of all notwithstanding the fact that 3 % cod-liver oil was added. It will also be observed that the mortality was very high with Group IV particularly during the 3rd and 4th weeks of growth. 50 % of these losses were the result of haemorrhage following the insertion of the identification bands into the wing.

Bleeding from the small wound slowly continued for 12 to 24 hours, the feather areas were continually wet with blood, the birds ultimately bleeding to death. The remainder of the losses all showed large sub-dermal haemorrhages along the femur (particularly the left), ribs and pectoral muscles and low blood volume due to haemorrhage. Blood from chicks in this group failed to clot on standing overnight in the laboratory. This condition was characteristic of the feeding of fish meal and to a less extent of meat meal which had been extracted with ether, no losses due to haemorrhage being recorded when caseinogen or untreated fish meal or meat meal was fed, although these chicks were all wing-banded at the same time and in exactly the same manner.

Coincident with the fact that the blood of the chicks fed ether-extracted fish meal would not clot there was also a distinct drop in the inorganic phosphorus content of the blood-serum (Fig. 3, Group IV) at the 4th week of growth. Unfortunately the losses in this group were so great that the blood-serum calcium and phosphorus determinations had to be discontinued at the end of the 4th week. However, the drop in the inorganic phosphorus is significant, particularly when it is noted (Fig. 3) that there was a very pronounced increase in the inorganic phosphorus content of the blood-serum during the 3rd and 4th weeks of growth of the chicks in all the groups fed unextracted fish meal. Since some factor other than the vitamin D, calcium or inorganic phosphorus content of the diet of the chick may have a profound effect on the concentration of inorganic phosphorus in the blood-serum during the first 8 weeks of growth, the value of this determination as a criterion of the presence or absence of rickets or leg-weakness in chicks is extremely questionable. It would seem that the removal of phospholipins from the fish meal was responsible for the result with Group IV but this does not explain the failure of the blood to coagulate, as the caseinogen of the control Group IX was also ether-extracted and the clotting time of the blood of the chicks was quite normal.

The results with these groups show that a diet containing approximately 15 % of this particular white fish meal as the only source of the fat-soluble vitamins contained sufficient vitamin A and vitamin D to give normal growth with no external evidence of either leg-weakness or rickets. The addition of 1 % cod-liver oil to this diet increased the rate of growth, presumably by increasing the vitamin A content of the diet, as increasing the concentration of vitamin D by ultra-violet irradiation had no significant effect.

With the meat meal groups (Fig. 2) the growth when no cod-liver oil was added (Group V) was extremely poor and all the chicks died within 5 weeks. The inorganic phosphorus content of the blood-serum of the chicks in this group fell rapidly during the first 4 weeks (Fig. 4, Group V). The chicks, however, did not grow sufficiently to show any external evidence of rickets. Giving the chicks ultra-violet irradiation resulted in an improved growth which, however, was still distinctly sub-normal and all the chicks were lost

within 6 weeks (see Fig. 2, Group VII). Adding 1 % cod-liver oil to the meat meal diet (Group VI), while greatly improving the results, still gave sub-normal growth when compared with the caseinogen control Group IX. Since normal growth was obtained with Group VIII receiving ether-extracted meat meal and 3 % cod-liver oil it does not seem probable that the nature of the protein or inorganic constituents was responsible for the sub-normal growth of Group VI. There is no significant difference in the composition of the ash of fish meal and meat meal so far as our analyses have been completed (see Table I). There were no external evidences of rickets in the chicks in Groups VI, VII and VIII. The inorganic phosphorus content of the serum of the chicks in the meat meal Group VI receiving 1 % cod-liver oil was constant throughout the first 8 weeks of growth.

The results with these groups show that a diet containing approximately 16·5 % of this particular meat meal as the sole source of the fat-soluble vitamins did not contain sufficient vitamin A or vitamin D to sustain growth. Since the addition of 1 % cod-liver oil to this diet still resulted in sub-normal growth and since normal growth was obtained when 3 % cod-liver oil was added to a diet containing ether-extracted fish meal it would appear that 1 % of high-grade medicinal cod-liver oil did not supply sufficient vitamin A or vitamin D to promote normal growth. Assuming the meat meal to be free from fat-soluble vitamins, the amount of vitamin A or vitamin D required to promote normal growth in chicks up to 8 weeks of age is, according to these results, in excess of the amount supplied by 1 % of high grade medicinal cod-liver oil.

Further work is being commenced to establish more accurately the vitamin A and vitamin D requirements of the chicks by varying the amount of cod-liver oil in the simplified caseinogen diet which has been used as a control in these experiments.

Summary.

1. A sample of white fish meal when fed at a level of 15 % in a diet composed of marmite and white rice was found to contain sufficient vitamin A and vitamin D to promote normal growth of chicks until 8 weeks of age. A sample of meat meal was found to contain little or no vitamin A or vitamin D when compared with fish meal.

2. The conclusion of Plimmer *et al.* [1927], that as little as 0·5 % of cod-liver oil of good quality is sufficient to rear chicks to maturity is based on the assumption that the fish meal used in their diets contained little or no fat-soluble vitamins. This assumption, for reasons discussed in the text, is believed to be erroneous. An experiment is described which shows that the amount of high grade medicinal cod-liver oil required to rear chicks, in the laboratory, to 8 weeks of age is in excess of 1 %.

3. Some factor, other than the vitamin D, calcium or inorganic phosphorus content of the diet of the chick, has been found profoundly to affect

the concentration of inorganic phosphorus in the blood-serum of chicks during the first 8 weeks of growth.

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XLV. THE MODE OF ACTION OF VITAMIN D. STUDIES ON HYPERVITAMINOSIS D. THE INFLUENCE OF THE CALCIUM-PHOSPHATE INTAKE.

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The production of toxic symptoms by overdoses of irradiated ergosterol was first recorded by Pfannenstiel [1927] and Kreitmair and Moll [1928]. A number of workers were unable to confirm this result [Dixon and Hoyle, 1928; Hoyle, 1929; Hoyle and Buckland, 1929; Comel, 1929; Cartland, Speer and Heyl, 1929; Lesné and Clément, 1929] and discredit was thrown by some on the theory of hypervitaminosis, it being suggested that the ill-effects in question must have been caused by toxic by-products, formed it was supposed from the alcohol used as solvent during the process of irradiation [Dixon and Hoyle, 1928; *cf.* Underhill, 1928]. Evidence in support of the opposing view that excess of vitamin D is toxic *per se*, so that the phenomenon genuinely merited the description of hypervitaminosis, was brought forward by Harris and Moore [1928; 1929, 1]. Without denying the possibility that secondary products might also be toxic, it was shown that the severity of the ill-effects ran parallel with the amount of vitamin ingested, that specimens of ergosterol irradiated in various solvents or no solvent were equally toxic when fed at the same level of antirachitic activity, that destruction of vitamin D by over-irradiation entailed a corresponding loss of toxicity, and that transformation products of ergosterol produced by resinification without irradiation were devoid of both antirachitic activity and of toxicity. These conclusions have since been confirmed by the experiments of other workers [*e.g.* Scheunert and Schieblich, 1929][2].

[1] In the whole time service of the Medical Research Council.

[2] Holtz and Schreiber [1930] similarly find that all preparations of irradiated ergosterol which display any antirachitic activity always possess a parallel degree of toxicity, and they are unable to obtain antirachitic but non-toxic products. On the other hand, they claim that they have been able to destroy the antirachitic activity and leave unimpaired the toxic (or "calcinosing") factor. However, the toxicity of the supposed non-antirachitic preparations was typified by its power of raising the blood-P or -Ca (*e.g.* in tetany) and (by definition) of producing calcification. Since these very effects are not infrequently taken as actual indices of antirachitic action, it is difficult to see how the positive result for toxicity, which was demonstrated by tests on dogs, can be reconciled with the reported negative result for antirachitic activity, obtained by tests on rats. Possibly the technique adopted (prophylactic method) may explain the anomaly. It may be

Perhaps the most convincing evidence as to the reality of hypervitaminosis accrued from a consideration of the very nature of the abnormalities produced. We argued [Harris and Moore, 1928; 1929, 1] that since in hypovitaminosis D (*e.g.* in rickets) the typical defective calcification at the growing end of the bone is associated with a diminished blood-P or -Ca, or both, so we might reasonably anticipate finding the reverse condition in hypervitaminosis D, where the characteristic lesion is an excessive calcification of the bone ending [Harris and Innes, 1928–1929], arteries, non-striated muscle[1], *etc.* [Kreitmair and Moll, 1928; Harris and Moore, 1929, 1]. The prediction was found to be justified, the administration of excess of vitamin D giving rise to hyperphosphataemia or hypercalcaemia or both in experimental animals [Harris and Stewart, 1929]. (This result has been confirmed in an extended series of tests [see Harris, 1930] and also by other workers. Hypercalcaemia or hyperphosphataemia has been seen likewise in infants receiving excessive doses of vitamin D [Hess, Lewis and Rivkin, 1928], while Hess, Weinstock and Rivkin [1928] had indeed earlier recorded that the blood-Ca reduced to a low level by a low-Ca, high-P diet could be brought back to a more normal figure by administration of very large amounts of irradiated ergosterol.) In the second place we suggested that just as the low blood-P or -Ca of rickets appeared to be associated with a diminished net absorption from the gut [see Harris, 1928] so again with very large doses of vitamin D an abnormally high net absorption might be expected[2]. Finally it was predicted that just as the severity of the hypovitaminosis is influenced by the Ca-P intake, so also this would probably prove to be a controlling factor in hypervitaminosis. These suppositions have proved to be justified, and a preliminary report showing that the severity of the hypervitaminosis is governed by the calcium intake appeared a year ago [Harris, 1930].

added that Windaus now employs the minimal lethal dose as a measure of the vitamin D activity of irradiated ergosterol products [Jephcott and Bacharach, 1930]. We must point out, however, how large is the number of workers who have been led to adopt the view that toxicity is associated entirely with by-products and not vitamin D [Adam, 1928; Reyer and Walkoff, 1928; Heubner, 1929; Steudel, 1929; Simmonet and Tanret, 1929, 1930; Holtz and Brand, 1929; Haendel and Malet, 1929; Vara-Lopez, 1930; Borghi, 1929]. A later view of Dixon [1929] and Hoyle [1930], that hypervitaminosis could only be produced on synthetic diets and not on a "natural" diet such as bread and milk, is accounted for in the present paper [see also Harris, 1930], where it is shown that the exact level of overdose of irradiated ergosterol needed to produce toxic symptoms varies with the Ca/P ratio of the diet.

[1] Similarly in rickets there is a deficient calcium content of muscle [Aschenheim and Kaumheimer, 1911; Haury, 1930].

[2] *Net absorption* = Ca [or P] intake *minus* faecal output = absorption from gut *minus* excretion into gut. In earlier papers the alternative phrase "retention from gut" was used in order to indicate that we were taking into account this factor of a possible excretion into the gut, *i.e.* that we were dealing with the "net" and not the "gross" absorption. The use of this phrase was perhaps unfortunate, since it may have led [*e.g.* Watchorn, 1930, 1] to confusion with the quite different concept of the retention by the animal as a whole (*i.e.* intake less urinary and faecal output). Of course we nowhere postulated that the retention as a whole was increased in hypervitaminosis, in fact we were the first to draw attention to the enormously increased urinary Ca excretion.

In the present communication our experiments, begun in 1928 and concluded in the spring of 1930, are described in greater detail and discussed more especially in relation to the mode of action of the vitamin. Convincing evidence is furnished to substantiate the theory that vitamin D exerts its action by raising the blood-Ca or -phosphate, so giving rise to an increased calcification. It is shown that the increase in the calcium and phosphate is associated with an increased net absorption from the gut (this is of special consequence in hypervitaminosis with diets rich in Ca), but also that the shaft of the bone may provide an important additional source under certain circumstances, for example on low Ca diets and with larger excess of vitamin D. The theory that vitamin D might function by drawing Ca and P out of the bone was put forward by Hess, Weinstock and Rivkin [1929, 2] and is supported by work in other laboratories carried out simultaneously with our own [Light, Miller and Frey, 1929; Hess, Weinstock and Rivkin, 1930; Baumgartner, King and Page, 1929; Brown and Shohl, 1930; Weinmann, 1929] as well as by that of Watchorn [1930, 2] in this laboratory. Jones, Rapoport and Hodes [1930, 2] on the other hand have concluded that the Ca comes from the food and not the bone. It does not appear to have been realised that these two methods of calcium mobilisation may both be involved, and to varying extents dependent on the Ca-P content of the diet and the degree of excess of the vitamin.

Experimental results.

All the experiments to be described here have been carried out on rats; we have obtained similar results with rabbits. The strain had been inbred for many generations, and carefully matched litter mates were always evenly distributed between the several groups in each experiment. Both piebalds and albinos (the latter originating from Wistar Institute stock) were used.

The irradiated ergosterol used in the later experiments[1] had an antirachitic activity of approximately 10^7 (Pharmaceut. Soc.—Med. Res. Council) units per g. and in the earlier experiments of approximately one-half to one-fifth as much.

In general, the degree of severity of the hypervitaminosis in a given experiment is reflected in a proportional impairment of growth, or loss in weight, so that the growth curves presented below convey a very fair impression of the relative toxicity of the various diets.

1. *Influence of variations in the calcium phosphate content of the diet.*

The same basal diet (Steenbock's rachitogenic diet [Steenbock and Black, 1925] with the calcium carbonate omitted) was fed to all groups, but with varying graded additions of calcium phosphate.

It was found (Fig. 1) that each increase in the calcium phosphate allowance gave rise to an increased severity of hypervitaminosis. By sufficiently re-

[1] We are indebted to Messrs J. Nathan and Co. for generous gifts of irradiated ergosterol.

ducing the calcium phosphate a level of vitamin D excess which was otherwise definitely toxic became virtually innocuous.

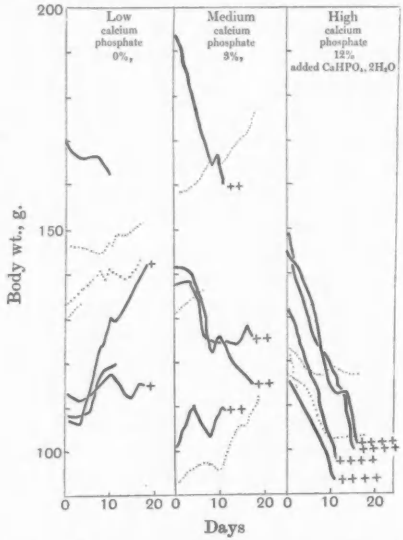

Fig. 1. Dependence of severity of hypervitaminosis D upon amount of calcium phosphate in diet.

\+ Microscopic calcium deposits (in kidney only).
\+ + Definite calcareous deposits.
\+ + + + Very extensive calcareous deposits.
—— 10 mg. irradiated ergosterol/rat/day.
..... Controls without vitamin D excess.

2. *Comparison of natural and synthetic diets with varying content of calcium phosphate.*

A series of tests was carried out to determine quantitatively the comparative effects of diets of Hovis bread and milk on the one hand, and synthetic diets, rich in calcium and phosphate, on the other, when fed in conjunction with various graded excesses of vitamin D. The results show that doses which are just on the border line of toxicity for the synthetic, salt-rich diet may be practically harmless for the bread and milk diets. However, when slightly larger excesses of vitamin D are given the difference becomes less noticeable, and with still larger excesses the difference virtually vanishes (Fig. 2). Next it was shown that the addition of an inorganic salt mixture (consisting principally of calcium salts and phosphates [Harris and Moore, 1928]) to the bread and milk diet sufficed to render it indistinguishable from a synthetic diet, in its power to permit hypervitaminosis at a given level of vitamin D excess (Fig. 3). Finally, we were able to demonstrate that on a diet consisting wholly of dried milk, and therefore very rich in Ca, an even more severe hyper-

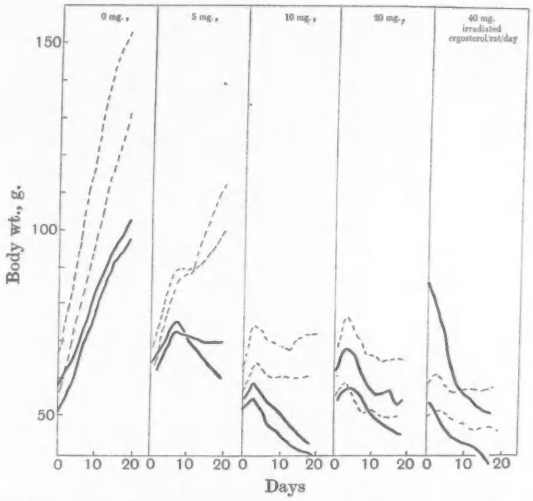

Fig. 2. Relative toxicity of vitamin D excess, on bread and milk and synthetic diets.
– – – – Hovis bread and milk (50:50).
——— Synthetic diet.

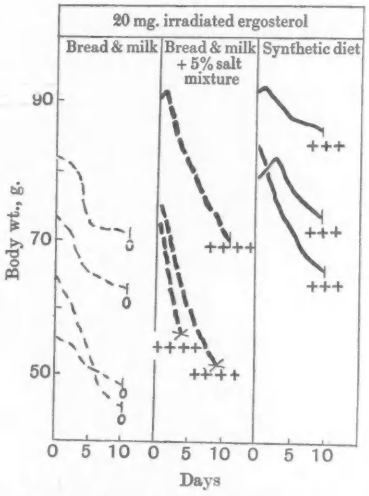

Fig. 3. Bread and milk diet *plus* added salt mixture permitting hypervitaminosis as readily as synthetic diet.

× Animal died. | Animal killed.
0 No calcareous deposits yet appeared.
+ + + Considerable calcareous deposits.
+ + + + Most extensive calcareous deposits.

vitaminosis was produced than on the usual synthetic diet, at the various graded levels of vitamin excess[1] (Fig. 4).

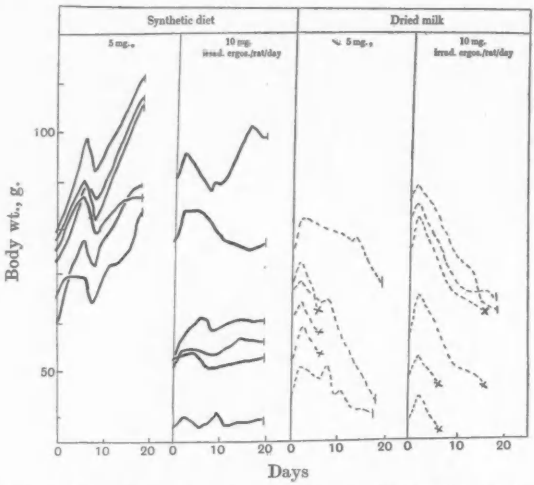

Fig. 4. Sole diet of milk even more favourable for production of hypervitaminosis than synthetic diet (4–5 % salts).
× Animal died. | Animal killed.

3. *Influence of variations in Ca/P ratio.*

These experiments were planned with the object of comparing the effects of large doses of irradiated ergosterol when administered in conjunction with

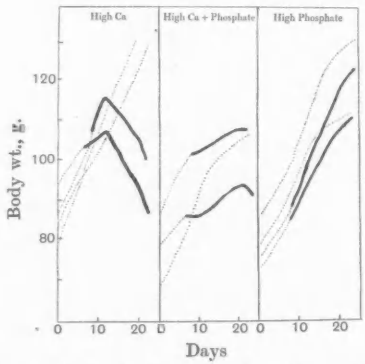

Fig. 5. Increased toxicity of irradiated ergosterol with increased Ca/P ratio of diet.
—— Irradiated ergosterol (0·05 % of diet).
..... Controls without vitamin D excess (1 drop "radiostol" per day).

[1] These results afford an explanation of the results of Dixon [1929] but fail to support his main conclusion:—"the same experiments made on animals living on an ordinary diet show that excess of the 'poisonous' food is harmless."

diets (1) rich in calcium but deficient in phosphate (2) rich and well-balanced with respect to both calcium and phosphate, and (3) deficient in calcium but rich in phosphate. The same basal diet was used throughout (based on Steenbock's formula [Steenbock and Black, 1925]: yellow corn, 60 g., gluten flour, 200 g., sodium chloride, 10 g.); but while the calcium-rich diet contained 38 g. of calcium lactate, the calcium- and phosphate-rich diet contained 30 g. of $CaHPO_4$, $2H_2O$ and the high-phosphate, low-calcium diet contained 24·8 g. of Na_2HPO_4.

Our results (Fig. 5) showed that a given overdose of irradiated ergosterol, just sufficient to cause rapid loss of weight with diets rich in calcium and deficient in phosphate, loses much of its toxicity when the calcium is balanced by the addition of phosphate, and that it becomes relatively harmless when the calcium is omitted and the phosphate remains high. This held true even when the feeding was prolonged for many weeks (Fig. 6). In further experi-

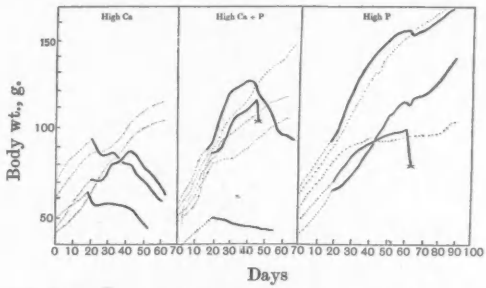

Fig. 6. Long-time feeding experiments, comparing high-calcium, high-phosphate, and high-calcium-*plus*-phosphate diets.
—— Irradiated ergosterol. ..... Controls.

ments (Fig. 7; *cf.* Fig. 8) in which the ergosterol was administered at higher levels or more fully-grown animals were used, a similar difference between effects of the high-calcium and the high-phosphate diets could still be detected.

Measurements of the calcium and phosphate metabolism of these animals are reported separately [Watchorn, 1930, 2]. The *post mortem* abnormalities characteristic for each group are discussed in Sections 6 and 7.

4. *Production of a distinctive type of hypervitaminosis on calcium-free or calcium- and phosphate-free diets.*

Since it had been shown (Figs. 5, 6, 7) that normal growth was permitted on high-P, low-Ca diets with such levels of irradiated ergosterol as were sufficient to cause rapid loss in weight and death when fed with diets containing both calcium and phosphate (or high-Ca, low-P), it was decided to ascertain whether even larger doses of irradiated ergosterol would still prove harmless under these conditions.

The results show that a toxic effect can be produced (at least eight times

as much irradiated ergosterol being needed when fed with a high-P, Ca-free diet as with a normal Ca-P diet, in order to produce loss in weight at an equal rate (Fig. 8)), but that there is a striking difference in the nature and

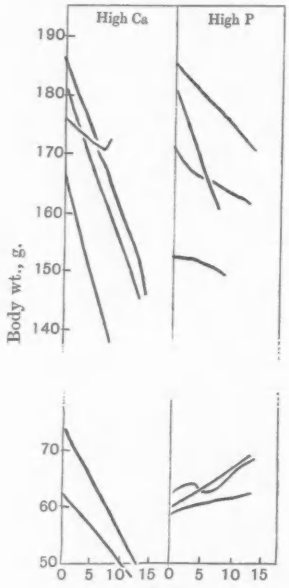

Fig. 7.

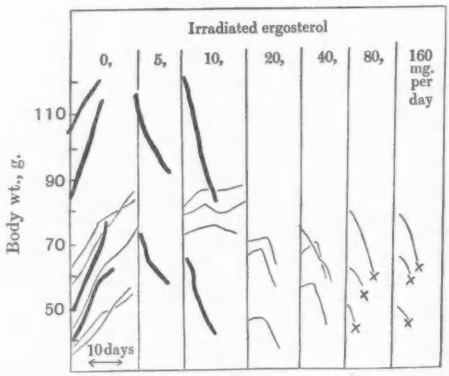

Fig. 8.

Fig. 7. Comparison of high-calcium and high-phosphate diets at higher level of vitamin D excess. Adults shown above. Representative curves from 50 animals.

Fig. 8. Hypervitaminosis induced on high-P, low-Ca diet by sufficiently increased vitamin D intake. —— Ca-deficient diet. —— Control diet, normal Ca and P. × Animal died.

symptoms of the hypervitaminosis in the two cases. Whereas the controls, on the diet containing both calcium and phosphate and 10 mg. irradiated ergosterol, showed the classical picture of hypervitaminosis, with calcification of vessels, heart and kidney, stimulation of bone formation, and, in the early stages, absence of noticeable osteoporosis in the cortex of the bone shaft or in the jaw bone, the experimental animals on the calcium-free diet with vitamin excess showed a rickety appearance in the bones, a pronounced resorption of compact bone together with almost complete absence of the usual calcification of vessels, kidneys, *etc.* In short, the changes in hypervitaminosis on the Ca-free diet resemble those on the control Ca-free diet without any vitamin excess, except that loss of mineral substance from the spongiosa is more extensive[1].

[1] The high-phosphate diet *per se* always tends to give rise to a characteristic kidney lesion, entailing focal necrosis of the tubules of the boundary zone, both in the hypervitaminosis and in the control groups.

Diets deficient in Ca and P. On a diet deficient in both calcium and phosphate, similar results were obtained (Fig. 9). Loss of weight and death resulted if sufficiently augmented doses of irradiated ergosterol were given, but

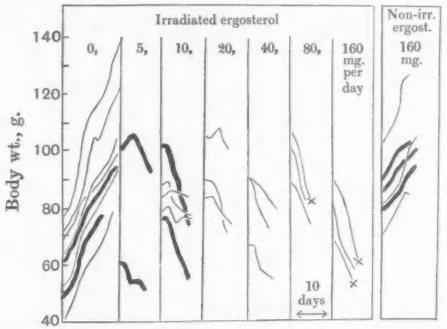

Fig. 9. Hypervitaminosis produced on diets deficient in Ca and phosphate.
—— Ca- and P-deficient diet. ——— Control diet, normal Ca and P. × Animal died.

no appreciable deposits of calcium salts had appeared in the organs, vessels or bone-endings, while the bones were soft and fragile with marked evidence of resorption and a rickets-like beading at the costo-chondral junction (again resembling the controls in the Ca- and P-deficient group without the vitamin D excess but with hastened resorption of spongiosa).

5. *Radiographs of bones.*

In view of the well-known effects upon bone structure of deficiency of vitamin D or of abnormal Ca-P intake, a detailed X-ray study was made of animals receiving excessive vitamin D, in the various groups and sub-groups already described[1]. The typical effects of hypervitaminosis upon the bony skeleton were best seen at the growing end of the long bone, and with diets containing adequacy of both Ca and P in normal ratio. Again the picture in hypervitaminosis may be contrasted with that in rickets (Plate I). In the early stage of hypervitaminosis, or when only moderate overdoses were given, an unmistakable narrowing of the metaphyseal cartilage was always seen. The epiphyseal end of the diaphysis became highly calcified, forming a characteristic dense band which rapidly broadened and extended to the marrow cavity. When the irradiated ergosterol was fed at very high levels of toxicity and the observations were continued for a sufficiently long period until the animal had lost weight considerably, it was found that the dense area continued to recede into the marrow cavity but left behind a distinct gap of lessened density, at the site which would normally correspond with the growing end of the bone.

[1] The exposures were made in the University Radiological Department, under the general supervision of Dr A. E. Barclay.

In contrast with the foregoing we found that when equally large overdoses of vitamin D were fed, but in conjunction with a diet such as is normally able to retard calcification (*e.g.* Steenbock's rachitic ration), excessive calcification of the bone-ending was in fact much less evident, or absent, although the production of calcareous deposits in the soft tissues (see below) was appreciably intensified.

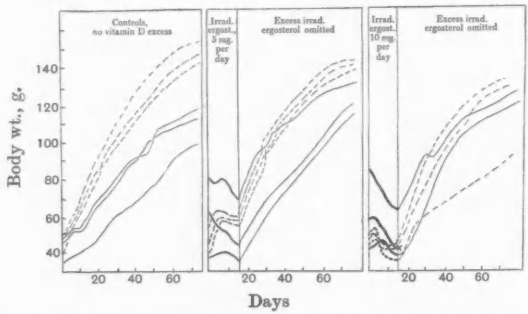

Fig. 10. Hypervitaminosis and cure (normal synthetic diet, and Steenbock rachitogenic diet).
Broken line, normal diet.
Continuous line, Steenbock diet.
Thickened line denotes excess of vitamin D.

6. *Factors favouring the formation of the calcareous deposits.*

An examination of our *post mortem* records for the various experiments described above makes it clear that the formation of the calcium deposits in the soft tissues (kidneys, arteries, non-striated muscle, *etc.*) is greatly increased when there is an abundance of calcium salts in the diet. As will be shown below the ingestion of large doses of vitamin D under such conditions gives rise to a greatly intensified hypercalcaemia (or hyperphosphataemia), associated with an increased net absorption from the gut. Examples of the influence of the calcium content of the diet in inducing calcareous depositions are seen in the following instances: (1) animals receiving Hovis bread and milk with 20 mg. of irradiated ergosterol lost weight rapidly but showed no calcium deposits after 10 days, whereas animals receiving the same diet *plus* 5 % added salt mixture had extreme and extensive calcium deposition, and animals on synthetic diet containing 5 % salt mixture showed fairly severe calcification; (2) after 12 days on 10 mg. of irradiated ergosterol and synthetic diet there were no naked-eye calcium deposits in animals given the low calcium phosphate diet, but there were definite calcium deposits in animals given medium calcium phosphate, and very extensive calcium deposits in animals given high calcium phosphate; (3) there was a virtual absence of calcareous deposits in animals having excess vitamin D with diets devoid of calcium, or devoid of calcium and phosphate (Figs. 8, 9); and (4) the calcium deposits were more readily produced on a diet of dried milk (Fig. 4) than on a diet

of bread and milk (Fig. 3). With the low-calcium diets, as will be shown, a relatively greater amount of calcium is drawn into the blood from the bone.

Two other factors concerned are the duration of the hypervitaminosis and the degree of excess of vitamin D in the diet. With insufficient excess, no calcium deposits are seen; with very large excess, the animal may die before the deposits have had time to develop. An animal may lose weight with hypervitaminosis for 15 days or longer before showing calcareous deposits (*e.g.* on the various diets referred to in Fig. 5; bread and milk, Fig. 3; etc.). Hence, the most favourable condition for the development of the calcareous deposits is a long-drawn-out hypervitaminosis, with no greater excess of vitamin D than is needed to cause a slow loss in weight, and an abundant supply of calcium salts in the diet[1].

7. *Histology of bone and teeth; osteogenesis and resorption in hypervitaminosis.*

The abnormalities seen in the bony skeleton[2] in hypervitaminosis D vary somewhat according to the stage of development, and also with the mineral salt content of the basal diet.

The effects seen with normal basal diets (complete synthetic diet; bread and milk; milk; *etc.*) may be described first. In the *teeth*, the most noteworthy feature is a remarkable overgrowth of cement, which extends to three or four times its normal thickness, shows much cellular proliferation and invades the marrow spaces of the jaw bone. The inner part of the dentine, to the extent of perhaps half its transverse thickness, is transformed into a distinctive type of secondary dentine, possessing a roughly granular and irregular laminated structure and staining intensely with haematoxylin (Plate II A).

In the *long bones* (or *ribs*) (Plate II B) the first change, observed with moderate over-doses of the vitamin and before toxic symptoms have appeared, is a marked stimulation of new bone formation. Osteogenesis is very active, and abnormally long and slender trabeculae are produced, extending far into the marrow cavity and already fusing to some extent. This is in conformity with the early radiograms (Section 5), which shows a dense shadow at the sub-epiphyseal region. At this stage the bones are solid and compact and there is little evidence of loss in density in the cortex of the shaft of the long bones or in the jaw bone. This is the picture seen in rats receiving bread and milk *plus* 5–10 mg. of irradiated ergosterol after 27 days (Fig. 2), or

[1] The contention that the calcium deposition in hypervitaminosis is subsequent to necrosis of the tissues is discussed elsewhere [Innes, 1930, *Ann. Rep. Dept. Animal Path., Camb.* (in press)].

[2] The specimens examined embrace (*a*) transverse sections of the lower jaw across the level of the root of the incisor teeth, (*b*) longitudinal sections of the costochondral junctions, and (*c*) longitudinal sections through the distal end of the femur and the proximal end of the tibia. These were fixed in formaldehyde saline and decalcified in 7 % HNO_3, embedded in paraffin in the usual way, and stained with haematoxylin and eosin.

synthetic diet *plus* 5 mg. irradiated ergosterol after 10 days (Figs. 8 and 9)[1]. As the process advances, the fusion of trabeculae continues so that a great mass of spongy bone is formed, which extends some considerable distance up the marrow cavity and occupies its entire width (Plate II B, no. 2). In the advanced degree of hypervitaminosis (Plate II B, no. 3), however, the most prominent feature is the evidence of excessive resorption. Macroscopically the bones become soft and fragile. The cortex of the shaft of the long bones and the jaw bone also, instead of remaining compact, is extensively osteoporosed and osteoclastic activity is prominent. Similarly a large marrow space appears at the epiphyseal end of the abnormal mass of spongiosa. This finding is in agreement with the radiograms taken during the late stages of hypervitaminosis, in which the dense shadow appears to have receded from the epiphysis. The picture here is complicated by the cessation of bony growth. The intermediate growth cartilage is very narrow and lined on the diaphyseal side by a thin transverse plate of bone. (This account applies, for example, to rats receiving bread and milk and the higher levels of irradiated ergosterol.)

The resorption from bone is much lessened when the diet is rich in calcium. Thus on a high-calcium, low-phosphate diet (Fig. 6), the animal may die with extensive calcareous deposits, but the bones and teeth will show no osteoporosis or other abnormalities, except those due to cessation of growth. On low-calcium diets, on the other hand, the resorptive changes become intensified. For example, the cortex is less compact on a bread and milk diet with hypervitaminosis than it is on a Ca-rich synthetic diet with the same excess of vitamin D (Fig. 2). Finally on Ca-free diets, or diets rich in phosphate and deficient in calcium, resorption is very extensive. A rickets-like appearance is seen resembling that in the controls on the same diets without the excessive vitamin, but in addition there is also a disappearance of the sub-epiphyseal spongiosa. Thus we have evidence of the bones acting as the main source of the extra calcium drawn into the blood stream when there is an insufficient supply from the gut.

8. p_H *of faeces.*

It has been shown that on certain rachitogenic basal diets the faeces of the rat tend to become abnormally alkaline when there is a deficiency of vitamin D, and to revert to a more normal reaction when the vitamin is restored to the diet [Zucker and Matzner, 1923; Jephcott and Bacharach, 1926, 1928; Bacharach and Jephcott, 1929; Grayzel and Miller, 1928]. An acid reaction in the gut favours solubility of Ca and hence ease of absorption. By analogy it seemed possible that feeding of excessive vitamin D might give rise to faeces with an abnormally low p_H figure. It has already been reported [Harris and Moore, 1929, 1] that, on an ordinary synthetic diet, no very decisive increase in acidity is in fact obtained. In continuation, we have examined the effects of excessive vitamin D given in conjunction with a

[1] These changes were not seen in adult animals where bony growth has ceased, but the formation of calcareous deposits was very marked.

Zucker diet [Jephcott and Bacharach, 1926], such as readily permits the change of faecal p_H in vitamin D deficiency[1]. Here again a similar result was found (Fig. 11).

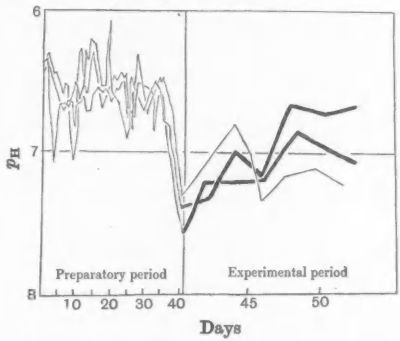

Fig. 11. p_H of faeces in hypervitaminosis.
——— Excessive vitamin D.
——— Control, or preparatory, diet (low in, or devoid of, vitamin D).

9. "*Vitamin balance.*"

In earlier papers [Harris and Moore, 1928; 1929, 1] we showed that the degree of severity of the symptoms of hypervitaminosis was sometimes diminished by an increase in the vitamin B (or B + C) allowance. This result has been fully confirmed [*e.g.* Duguid, Duggan and Gough, 1930].

A somewhat similar phenomenon was the production of symptoms resembling avitaminosis B by feeding excessive cod-liver oil [Harris and Moore, 1928] or cod-liver oil concentrate [Harris and Moore, 1929, 2]. These symptoms could be cured or prevented by a slight increase in the vitamin B allowance. The same result has since been obtained independently by American workers [Norris and Church, 1930]. However, we thought it necessary to emphasise that this balancing effect could only be readily seen when the vitamin B allowance was already low, and the cod-liver oil excess very considerable.

Further work on hypervitaminosis D confirms the view that similar conditions apply here. If the diet is already rich in vitamin B, further small additions fail to show any appreciable alleviating effect in protecting the animal against massive overdoses of vitamin D (Fig. 12). It is clear therefore that in interpreting such results, a limited significance only can be attached to the conception of a balance between vitamins D and B.

Discussion.

The observations recorded in the above experimental section, taken in conjunction with results described in earlier papers, appear to furnish us with

[1] The electrometric determinations of p_H were kindly carried out by Miss E. Allchorne of the Glaxo laboratory.

a logical and consistent picture of an orderly sequence of changes induced by the action of vitamin D in the animal organism. As we pass from a deficient intake of vitamin D to an adequate level, then to an abundance, and so to excess, and finally to very great excess, a series of phenomena is seen in increasing intensity. Our main deductions may first be summarised briefly as follows, and we can then proceed to a more detailed critical analysis. An increased vitamin D intake promotes increased absorption of Ca and/or phosphate from the gut (or diminished excretion into the gut), so tending to raise the level in the blood. With inadequacy of vitamin D, the blood fails to

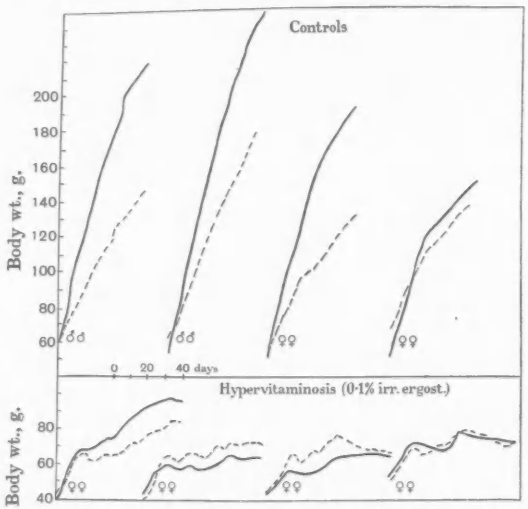

Fig. 12. Hypervitaminosis on (1) synthetic diet, and (2) with the sugar (60 % of diet) replaced by equivalent of concentrated orange juice (note remarkable growth rates of controls on the orange juice regimen).

– – – Orange juice diets } matched litter mates.
——— Synthetic diets }

secure sufficient Ca and/or phosphate; with moderate amounts the blood succeeds in maintaining its approximate constancy of composition; while with large overdoses, hypercalcaemia and/or hyperphosphataemia cannot be averted. In the latter condition the kidney responds by an abnormal urinary excretion of Ca and/or P. If on the other hand the excessive vitamin D be now suddenly removed, a suitably increased excretion of Ca and phosphate is permitted to the gut [Watchorn, 1930, 1]. The "retention" of Ca or phosphate by the animal as a whole is seen to be the resultant of two factors, working in opposite directions, *viz.* (i) the increased absorption from (or diminished excretion into) the gut, and (ii) the increased excretion by the kidney. As more and more vitamin D is given the latter factor overtakes the former, so that the resulting retention, while it is increased by moderate doses

Page 380. Key to Fig. 12

for ------ Orange juice diets
——— Synthetic diets

of the vitamin, is diminished by very large doses. The deposition of calcium salts in certain sites is associated with a withdrawal from others; in hypervitaminosis this transference process in the bone is apt to be excessive; in rickets there is evidence of a defective transfer [Morelle, 1930]. As has been shown in the experimental section, when the diet is deficient in calcium the withdrawal from the bone stores becomes the noteworthy feature of the hypervitaminosis. When on the other hand the diet is rich in calcium the bone is less called upon, but there is increased liability to calcareous deposition as a result of an increased net absorption from the gut, and partly also no doubt because the amount stored by the bone is less than normal. Each addition of calcium to the diet intensifies the hypercalcaemia and calcareous deposition with a given overdose of vitamin D.

It is clear, therefore, that the precise level of Ca and P in the blood as influenced by the vitamin D intake depends on a greater variety of complex factors than has been generally realised in the past, including gut absorption (or excretion), kidney excretion, and deposition and dissolution of calcium deposits.

The experimental results are not inconsistent with the simple view that vitamin D acts by increasing the apparent solubility of the Ca and phosphate in the blood, an increased content in the blood facilitating deposition in the sites to be calcified. The latter assumption seems justified in view of the observation [Shipley, 1924; Shipley, Kramer and Howland, 1926] that non-calcified cartilage from a rachitic rat will rapidly calcify once it is put in blood-serum or inorganic saline containing adequate Ca and P (or Ca × P product). Our evidence also indicates that vitamin D can serve to promote osteogenesis. This could be explained readily enough, however, by supposing that the rate of formation of fresh osteoid tissue depends on the rate at which the old becomes calcified, in which case clearly the precipitation of extra calcium would mechanically serve to stimulate fresh osteogenesis.

It is obvious that much information has still to be acquired before a full explanation can be given for the preferential deposition of calcium salts in certain types of tissue to the total exclusion of others; this is seen as well in hypervitaminosis as under normal physiological conditions. The presence of phosphatase [Martland and Robison, 1924] is doubtless of special importance, so too perhaps the alkalinity.

The suggestion that vitamin D mobilises Ca (or P) by stimulating the parathyroid demands consideration. Obviously the theory could be tested by determining the influence of vitamin D after parathyroidectomy. The results of such tests hitherto recorded are very conflicting [Hess, Weinstock and Rivkin, 1929, 1, 1930; Jones, Rapoport and Hodes, 1930, 1; Taylor, Branion and Kay, 1930]. Nevertheless it seems probable that vitamin D can function independently of the parathyroid, for the parathyroid hormone raises the calcium in the blood solely by withdrawing it from the bones and without increasing the net absorption from the gut.

Certain points in the main conclusions outlined above appear to run counter to current views or to recent observations by other workers, and the evidence in their favour will now be analysed in greater detail.

Gut absorption.

The measurements taken by Miss Watchorn of calcium and phosphate intakes and faecal outputs upon the animals dealt with in Fig. 7 (Steenbock basal diet, (1) with added calcium and no phosphate, and (2) with added phosphate and no calcium) appear at first sight to contradict our assumption of an increased stimulation of gut absorption through the agency of vitamin D. However, in interpreting her results [Watchorn, 1930, 2], several complicating factors have to be borne in mind, most notably that of starvation, or general failure. Once a severe degree of hypervitaminosis has supervened (so that the animal is consuming only a very small fraction of its normal food intake and body processes are failing and perhaps calcium deposits already appearing in kidneys, muscles, vessels, intestinal tract, *etc.*) the gut function appears to break down and the effect of vitamin D in increasing the absorption can now no longer be demonstrated. This was illustrated clearly enough in Watchorn's earlier paper [1930, 1], in which she showed, working with normal basal diets, that vitamin D excess at first caused an increased net absorption (in every instance but one), particularly when the absorption had previously been low, but that this absorption ultimately fell off, as the animal ceased to eat. Partial starvation, in fact, was shown in a control experiment to be sufficient in itself to diminish Ca and P absorption. If now we review in the light of these considerations the results with the Steenbock high-Ca diet [Watchorn, 1930, 2], we find that the analyses refer to the period when these very complications had already set in and the animals were losing weight and taking very little food (in the second week of hypervitaminosis). An unequivocal demonstration of increased net absorption could not therefore be expected. Even so, it can be shown that some effect on the Ca is still apparent, when figures are recalculated upon a percentage basis, in the case of two animals out of the three. The third animal may be fairly neglected for its food intake had dropped 75 %, compared with only 46 % and 54 % for the other two, and absorption from the gut had indeed virtually ceased.

The phosphorus absorption in this test showed immense fluctuations between the three animals in the group, varying from 56 to 77 % in the preliminary period, from 64 to 15 % in the hypervitaminosis period, and from 57 to 15 % in the recovery period[1]. However, a similar investigation has been carried out by Brown and Shohl [1930], but embracing a series of different levels of vitamin D feeding; and since their results have the advantage of showing greater uniformity than those just discussed, it has been thought

[1] This relates to the organically bound P of the phosphate-free Steenbock diet. Since in vitamin D metabolism we are primarily concerned with inorganic phosphate, the meaning of the figures might conceivably be disputed, but the results of Brown and Shohl (*v. infra*) definitely show its increased absorption under the influence of vitamin D.

worth while to recalculate their figures, also upon a percentage basis, in order to demonstrate the remarkable consistency and regularity with which each increase in vitamin D does in fact give rise to an increased net absorption from the gut, until finally the toxic complications supervene. Thus on Steenbock's rachitogenic diet (high-calcium, low-phosphate) the addition of 10,000 times the minimal effective dose of vitamin D increased the net absorption of phosphorus by no less than 300 %, and of Ca by about 50 %. With still larger doses, and the appearance of toxic symptoms and loss of appetite, the absorption begins to fail (Table I). Brown and Shohl themselves, although drawing attention to the increased urinary elimination of Ca and P with large doses of vitamin D (the excretion as they say shifting from the faeces to the urine, and the retention decreasing), did not attach special significance to the question of gut absorption, in fact they stated that the amount of Ca and P excreted remained relatively constant (in the absence of toxic symptoms).

Table I. *Increase in net absorption of Ca and P resulting from increasing doses of irradiated ergosterol. Recalculated from Brown and Shohl [1930]. (Steenbock high-Ca, low-P diet).*

Daily dose of irradiated ergosterol (mg.)	0	0·01	0·1	0·5	1·0	2·0
P "absorbed," % of intake	8, 12	22, 27	26, 26	33, 37	41, (21)*	(27)*
Ca "absorbed," % of intake	31, 36	42, 43	43, 49	48, 48	49, (34)*	(34)*

* Marked loss of appetite.

A similar examination of Brown and Shohl's figures for a diet (Sherman) with a normal Ca/P ratio, as opposed to the rachitogenic, high-Ca, diet, shows a corresponding if less intense effect. An increase of vitamin D by about 2000 times above the minimal protective dose gives rise to a well-marked increase in the weight of Ca and P absorbed (Fig. 13), while at higher levels the complicating influence of the lowered food intake is again evident, even when results are calculated on a percentage basis (Fig. 14).

With our diet rich in phosphate but free from Ca (Fig. 7) the net absorption of P was already at so high a level (83 to 93 %) in the control that the demonstration of any increase under the action of vitamin D was not to be anticipated. It may be recalled indeed that at the level of vitamin D excess employed no toxic ill-effects were seen with this diet. As has already been suggested the effect on phosphate may be secondary to that on Ca.

But perhaps the most striking argument that can be deduced in support of our view, that large doses of vitamin D tend to raise the absorption of Ca and phosphate (or to diminish their back excretion into the gut), is obtained from a study of the effects of a cure in hypervitaminosis. An animal having the typical abnormally high blood-phosphate or -Ca induced by excessive doses of vitamin D is suddenly put back on to a diet free of any such excess. The curative process begins immediately. The absence of vitamin D, it can

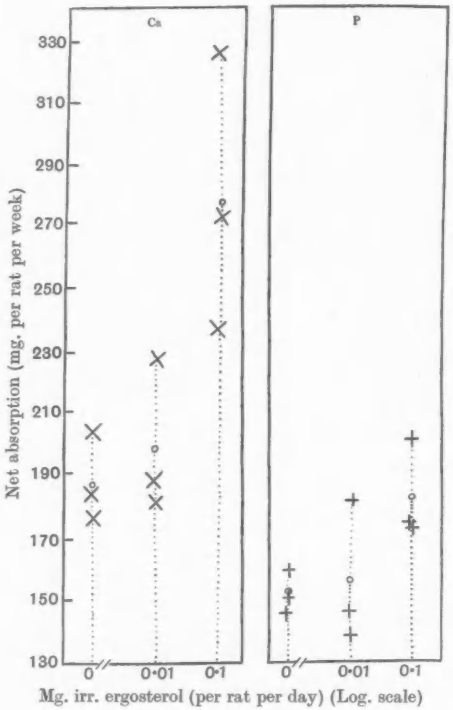

Fig. 13. Increase in Ca and P net absorption with increasing doses of irradiated ergosterol. Recalculated from Brown and Shohl. Normal diet (Sherman).
0 = mean readings.

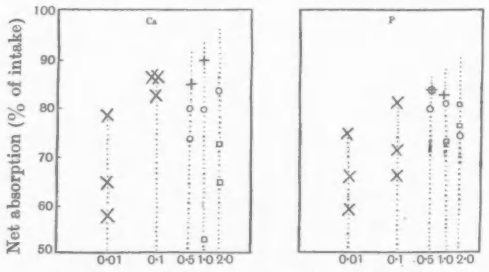

Fig. 14. Complicating influence of failure and loss of appetite with high levels of toxicity.

×	Intake approx. normal,	240–390 mg. Ca per week.	
+	„	below normal, 170	„
○	„	low, 110–150	„
▫	„	very low, under 100	„

be shown, now permits the animal to excrete in its faeces a vastly increased amount of Ca or P, more suitable to the excessive blood level and tending to reduce it towards the normal. The faecal calcium now reaches figures as high as 65–90 %, compared with only 10–20 % in the presence of the excessive vitamin D, or 20–60 % for the appropriate controls (expressed as percentage of intake) (Fig. 15). Thus the point of view we are here advocating will allow

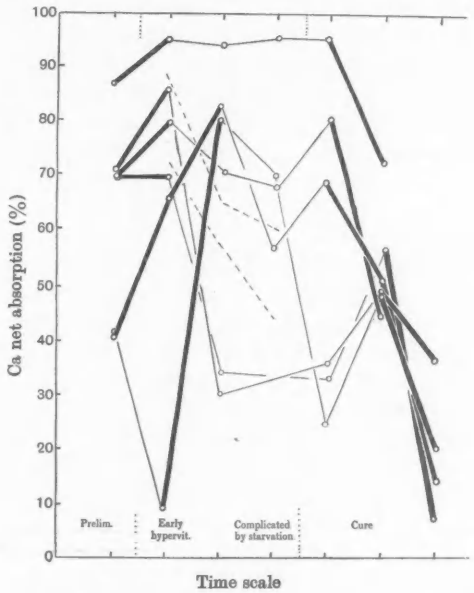

Fig. 15. Rise in net absorption at beginning of hypervitaminosis, complicating influence of starvation, and fall in cure.
– – – Starvation control without hypervitaminosis.

a rational interpretation of the underlying causes of the "even graver disturbances in absorption and retention during the recovery period," to which attention was first drawn by Watchorn [1930, 1].

We have therefore the following evidence pointing to increased net absorption under the influence of vitamin D:

(1) the dependence of the hypercalcaemia and calcareous deposition in hypervitaminosis D upon the Ca intake;

(2) the occurrence of a greatly increased faecal excretion when the hypervitaminosis is terminated;

(3) direct calculations upon data for intakes and faecal outputs, including the earlier uncomplicated stages of hypervitaminosis;

(4) measurements by earlier workers upon clinical and experimental hypovitaminosis and its cure.

Raised blood-Ca and/or -phosphate.

The production of hypercalcaemia and/or hyperphosphataemia in rabbits or rats by the action of excessive doses of vitamin D, first reported by Harris and Stewart [1929], and repeated under a wider variety of conditions [Harris, 1930], has been repeatedly confirmed, *e.g.* by Ashford [1930] who showed that there was no concurrent increase in the organic acid-soluble phosphate, and the observation has been extended to other species including dogs [Jones, Rapoport and Hodes, 1930, 1], chickens [Massengale and Nussmeier, 1930], and cows [Greig, 1930]. But certain workers have obtained less consistent or even negative results, the reasons for which demand consideration here.

In the first place the important consideration must be borne in mind, as originally indicated [Harris and Stewart, 1929] and again confirmed in our present results, that the severity of the condition is largely governed by the amounts of calcium or phosphate ingested. The influence of this factor is clearly apparent also in the recent result of Warkany [1928], who showed that the rise in blood-phosphate following a meal of inorganic phosphate is greatly increased by the administration of irradiated ergosterol. In the second place it must be realised that an increase in both Ca and P simultaneously is not necessarily seen; sometimes one is affected first, sometimes the other. The influence of calcium equilibrium upon phosphate and *vice versa* is apparent here. Next, the hyperphosphataemia or hypercalcaemia, varying as it does with the diet, is to be understood in a relative rather than an absolute sense; for example, the figures of Shohl, Goldblatt and Brown [1930] show that in rats fed on high-phosphate or high-calcium diets with excess vitamin D, the blood-Ca or -P is high compared with the corresponding controls, although not necessarily of course compared with the normal rat. Finally, negative results have sometimes undoubtedly resulted from failure to give sufficiently large excess of the vitamin. It was earlier suggested [Harris and Moore, 1929, 1, p. 272] that such an explanation might account for the observations of Havard and Hoyle [1928], who, working on man, were unable to obtain any rise in inorganic P or serum-Ca above the low winter level. Confirmation for this view appears to be found in more recently published work on human beings, by various authors, in which very high levels of vitamin D have been fed, and one or other of the following typical abnormalities reported, *viz.* hypercalcaemia, or hyperphosphataemia, or increased renal excretion of P or Ca, or overcalcification of bone-endings, and, even, calcareous deposits [Hess, Lewis and Rivkin, 1928; György, 1929; Puschler, 1929; Hughes *et al.*, 1929; Hottinger, 1929; Kroetz, 1927; Lasch, 1928; Ghirardi, 1929].

Withdrawal of calcium from bone.

Our results show that with diets very rich in calcium a relatively small excess of vitamin D is sufficient to cause hypercalcaemia (or hyperphosphataemia) and the appearance of calcareous deposits. Under these circum-

stances there is, of course, little evidence of osteoporosis. Similarly, chemical analysis showed that there was no appreciable change in the mineral content of the bone. These results were published in a preliminary communication which stressed the importance of the Ca intake as a determining factor in hypervitaminosis; but unfortunately the following statement, couched in far too sweeping terms, was made, "we find by chemical analysis that there is no loss of Ca or P from the bones to account for the rise in the blood figure" [Harris, 1930]. It should have been made clear that this related only to these particular diets. With very high doses of vitamin D, particularly on Ca-deficient diets, the withdrawal of mineral from the bone becomes the outstanding feature. (This conclusion was also reached indirectly [Watchorn, 1930, 2] from analyses of faeces and urine for the animals shown in Fig. 8.) The evidence of a similar withdrawal and re-deposition in the cure of rickets has already been alluded to.

Abnormalities in bone structure in hypervitaminosis.

In marked contrast with our evidence of immense stimulation of spongiosa formation and resorption of compact bone, Shohl, Goldblatt and Brown [1930] found "no striking abnormalities" histologically in the ribs in hypervitaminosis. On the other hand in the recent independent work of several other investigators [Kreitmair and Hintzelmann, 1928; Baumgartner, King and Page, 1929; Weinmann, 1929; Collazo, Varela and Rubino, 1928, 1929, 1, 2, 3] many conclusions may be found in common with our own. Hypercalcification of the growing end of the bone was also clearly shown in radiological observations on children by Hess, Lewis and Rivkin [1928], and on rats by Shohl, Goldblatt and Brown [1930], and by György [1929].

Urinary calcium (or phosphate) excretion.

Harris and Stewart [1929] found that in hypervitaminosis D the renal excretion of calcium was so much increased that the urine became cloudy with calcium salts. They interpreted this to mean merely that the organism was endeavouring to dispose of the excessive calcium carried into the blood stream. More recent quantitative tests have abundantly confirmed the existence of the remarkably high urinary output either of phosphate or of calcium or both [*e.g.* Ashford, 1930; Watchorn, 1930, 1, 2; Brown and Shohl, 1930] and the negative result of Hoyle and Buckland [1929] must presumably be ascribed to the use of insufficient excess of vitamin.

Summary.

A study has been made of the influence of variations in the calcium and phosphate intake upon the abnormalities resulting from overdoses of irradiated ergosterol. An increase in the calcium content of a diet (or in the Ca/P ratio) intensifies the severity of the hypervitaminosis and gives rise to

an increased formation of the calcareous deposits, at a given level of vitamin D excess. With diets virtually devoid of calcium and phosphate, on the other hand, a hypervitaminosis of a distinctive character can still be produced provided now that the level of vitamin D excess is sufficiently raised; under these conditions calcareous deposits are not in evidence but there is a greatly increased resorption of bone substance.

X-ray and histological examinations of the bones have shown that large doses of vitamin D (with normal diets) stimulate osteogenesis, and a densely calcified overgrowth appears at the growing end of the bone (in contrast with rickets); while in the advanced degrees of the hypervitaminosis resorption is extensive, and the cortex of the shaft and other "compact" bone becomes spongy. Vitamin D excess also gives rise to a remarkable overgrowth of cement in the growing animal.

In explanation of these and earlier results it is shown that feeding of excess of vitamin D (in the various types of hypervitaminosis examined) gives rise to a raised blood-calcium or -phosphate or both, with a tendency to deposition of calcium in certain sites, just as vitamin D deficiency (*e.g.* in rickets) gives rise to lowered blood-phosphate or -calcium or both and accompanying inadequate calcification. The extra calcium and phosphate may be derived in two ways: (1) increased net absorption from the gut; and (2) increased withdrawal from bony stores, vitamin D therefore having a distributive action. In the hypervitaminosis produced on diets rich in calcium with moderate overdoses of vitamin D the first of these factors is of special consequence, while with Ca-deficient diets and with larger excesses of the vitamin, withdrawal from the bone shaft is the main source.

That vitamin D increases absorption of Ca (or P) from the gut (or decreases excretion into the gut) is shown: (1) from the effects of increased Ca intake in hypervitaminosis, (2) from the observation that withdrawal of vitamin excess causes greatly increased faecal excretion of Ca (or P), (3) from recalculations of available data for intake and faecal output, it being shown that each addition of vitamin D to a diet causes an increased net absorption, until ultimately with highly toxic levels a generalised failure complicates the result, and (4) from evidence from rickets and its cure. The mode of action of vitamin D in this respect stands in contrast with that of the parathyroid hormone.

Thus while addition of vitamin D to the diet tends to raise the blood-Ca (or -P, since one influences the other) the actual level so attained is the resultant of several factors: operating in one direction is the increased net absorption and dissolution from certain sites, and, in the opposite, the deposition in other sites, and (when high levels are reached) an increased urinary excretion. With increasing doses of vitamin D the retention by the animal as a whole first rises but ultimately falls, the kidney excretion overtaking the gut absorption.

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DESCRIPTION OF FIGURES IN PLATES I AND II

PLATE I. Comparative X-ray photographs showing end of long bone (knee joint), (1) normal, (2) in rickets, and (3) in hypervitaminosis.

C = epiphyseal cartilage. *E* = epiphyseal end of diaphysis.

1. Normal.

2. Rickets: widening of epiphyseal cartilage and deficient calcification of epiphyseal end of diaphysis.

3. Early hypervitaminosis, 8 days: abnormally narrow epiphyseal cartilage; and broad dense band of excessive calcification at epiphyseal end of diaphysis, extending into marrow cavity.

4. Later stage, 14 days (complicated by cessation of growth and osteogenesis): appearance of a gap between the dense overcalcified area and the epiphyseal cartilage.

5. Same, more advanced, 29 days, with calcification at epiphyseal cartilage *C*.

6. Cure of early hypervitaminosis: (*a*) partial loss of excessive calcium, (*b*) cure complete, bone structure returned to normal.

7. Hypervitaminosis with same level of vitamin D excess but on Steenbock's rachitogenic diet: bone structure almost normal.

PLATE II. Bones and teeth in hypervitaminosis D (rat).

A. TEETH.

1. *Normal control.* Note uniform normal dentine (D), cement (C) and jawbone (J). (Transverse section of incisor. Haematoxylin and eosin. ×160/3.)

2. *Hypervitaminosis in young rat.* Enormous overgrowth of cement (C). Formation of true bone (b) near dentinal junction. Osteoporosis of jawbone (J). Abnormal secondary dentine (D). (×160/3.)

B. LONG BONE.

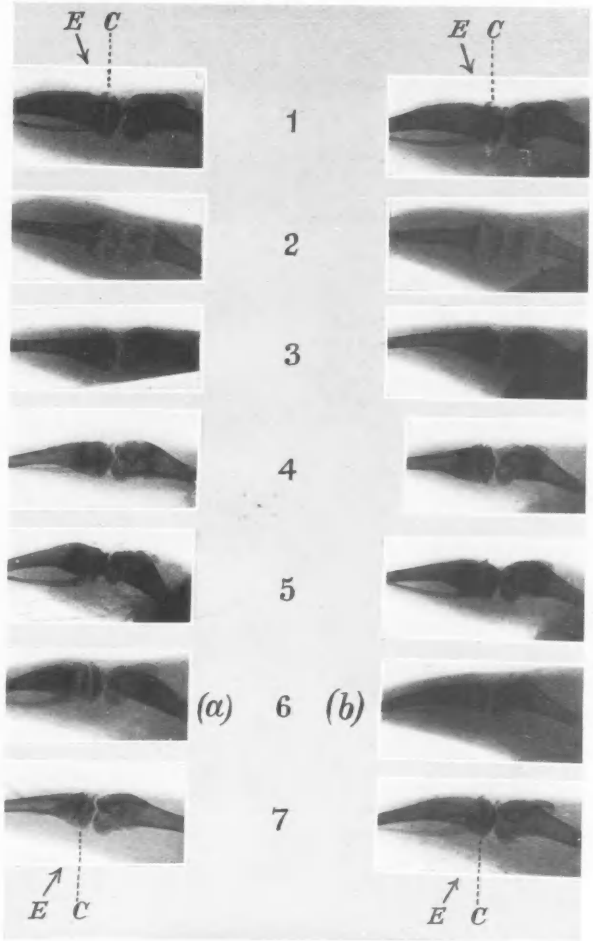
1. *Normal control.* Note features of normal osteogenesis at epiphysis: regular intermediate growth-cartilage (C), showing normal columns of cartilage cells and trabecular formation (T). Normal compact cortex of shaft (S). (Distal end of femur. ×50/3.)

2. *Early degree of hypervitaminosis* before much resorption had occurred from cortex of shaft (no severe calcification of kidney or aorta was yet apparent at *post mortem*). Decrease in thickness of intermediate growth-cartilage (C). Increased new bone formation: trabeculae covered with thick lamellae of bone (T). (Proximal end of tibia. ×50/3.)

3. *Later stage.* Thin intermediate growth-cartilage (C). Further great increase in subepiphyseal spongiosa (T) extending far into marrow cavity. Thin osteoporosed cortex of shaft (S). Slight resorption (R) of spongiosa nearest growth cartilage. (Distal end of femur. ×50/3.)

Page 390. Lettering on Plate II, A, in figs. 1 and 2:
"C" should be raised $\frac{1}{8}$″ and $\frac{1}{2}$″ respectively.

Plate II, description of A, fig. 2:
for **Enormous overgrowth of cement (C). Formation of**
true bone (b)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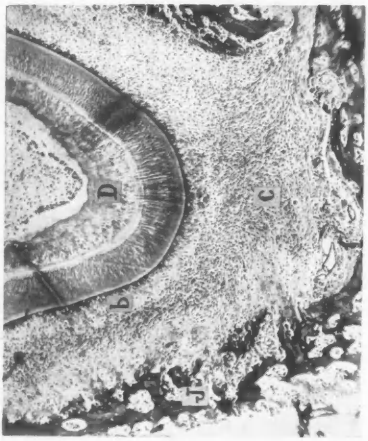

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XLVI. THE ELECTROPHORETIC VELOCITIES OF GELATIN AND OVALBUMIN IN DIFFERENT CONCENTRATIONS OF THEIR MIXTURES AND THE EFFECT OF ULTRA-VIOLET IRRADIATION.

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(*Received January 1st, 1931.*)

Observations of the motions of proteins in an electric field by many different methods have given results which, as shown in a previous paper [1929], are in substantial agreement. In this communication we have described the conditions under which the motion of a boundary, made visible by means of colloidal gold, may be measured in an improved form of U-tube apparatus [1930].

The investigation has now been extended from 0·1 % solutions of proteins to those mentioned in the title of this paper. In these solutions, differences in potential gradient are enhanced, and inconstancies in the motions of the boundaries are more frequent. The causes of these inconstancies, as they affect hydrophobic sols such as those of metals and sulphides, have been considerably elucidated by the work of Mukherjee [1923] and Kruyt [1925; Kruyt and van der Willigen, 1928].

Mukherjee used a U-tube of the type first described by Burton [1906] in which the sol is run in below the sol-free electrolyte. Many workers have found it difficult to set up a sharp boundary in such a manner. The difficulty has been obviated, for example in our apparatus, by separating the sol from the upper solution of electrolyte by means of taps, which are only opened after complete hydrostatic equilibrium has been established between the two limbs. Also disturbances due to diffusion of the products of electrolysis from the electrodes to the moving boundary are encountered when using the Burton type of tube. These were avoided in our apparatus, and in many others, by setting the electrodes in side-tubes. Any outstanding irregularities in Mukherjee's results are probably due to these causes.

This worker measured potential gradients in various sections of the tube by means of side-tubes sealed in at different levels, and found considerable variations when an arsenious sulphide sol containing free HCl and H_2S was introduced below a KCl solution having the same conductivity. When, however, HCl, having a slightly greater conductivity than that of the sol, was placed above the latter, the potential gradients in the sections immediately

above the original position of the boundary remained nearly constant during an experiment. These potential gradients gradually increased from the top section (HCl) to the lowest section (HCl + sol + H_2S). The average velocities in the lower sections, each corrected for their potential gradients, were in good agreement. They were 3·74 and 3·84 μ/sec., v./cm. In the anode limb, where the specifically slower SH^- ion follows the faster Cl^- ion upwards, the boundary remains sharp, while in the cathode limb where the faster ion follows the slower, the boundary becomes diffuse. Yet the calculated velocities in each limb were found to be nearly equal. These results seem to show that, although ionic diffusing layers may mix up the migrating particles, these do not take part in the ionic motion to the extent of being accelerated by the faster ions, *i.e.* the particles do not behave in this respect like slow ions. In agreement with this, it was shown that equivalent concentrations of sulphuric acid gave the same electrophoretic velocities, although there is considerable difference between the mobilities of Cl^- and of $\frac{1}{2}SO_4^=$. Although this conclusion does not necessarily hold good for a protein micelle, we may assume that it is approximately true for low velocities of the protein.

The general results of Mukherjee may be summarised:

(*a*) The velocity of movement of a boundary is dependent on the potential gradient of the section of tube in which it is moving.

(*b*) This potential gradient is determined by the conductivity of the solution in each section, including any conductivity due to the sol particles themselves.

Kruyt and van der Willigen [1928] also investigated sols of As_2S_3 containing up to 22 g. per litre. On these were superimposed intermicellar liquids obtained by ultra-filtration of the sol through collodion. The conductivities of the sols were 2–3 times those of the intermicellar liquids, partly owing to the decomposition of H_2S in the latter, partly owing to the presence of the negative sol particles in the former. The observed velocities in the "down" and "up" columns (cathode and anode limbs) were divided by the potential gradients in the sol and intermicellar liquid respectively, and agreed well. Thus the velocities of a sol containing 17·5 g. As_2S_3 per litre were 4·18–4·25 μ/sec. v./cm. at 20°. The potential gradients E_1/l_1 and E_2/l_2 in the sol and solution columns may be calculated from the conductivities, since

$$E_1/l_1 : E_2/l_2 = \kappa_2/\kappa_1.$$

Thus, in one case, $E_1/11{\cdot}5 : E_2/12{\cdot}1 = 6{\cdot}21/16{\cdot}15$

and E_1 and $E_2 = 75$ v.

Therefore $E_1/l_1 = 1{\cdot}75$ and $E_2/l_2 = 4{\cdot}55$ v./cm.

The observed velocity on the upward side was 1·35/900 cm./sec., therefore the velocity under unit potential gradient was

$$\frac{1{\cdot}35 \times 10^4}{900 \times 4{\cdot}55} = 3{\cdot}3\ \mu\text{/sec., v./cm.}$$

On the assumption that the velocity is determined solely by the potential gradient, another formula may be deduced which gives the velocity "v" in terms of the observed velocities v_1 (up) and v_2 (down), *i.e.*

$$v = \frac{v_1 l_1 + v_2 l_2}{E}.$$

We have used this to check some of our results.

It seems probable that the difficulties of determining electrophoretic velocities of hydrophobic colloids have been completely overcome.

As a provisional method of attack and pending a more complete knowledge of the complex protein micelles, it seems justifiable to apply these results to systems containing proteins, bearing in mind that, by virtue of their amphoteric character, these may behave to a greater or less extent as multivalent ions of rather low mobility.

Experimental.

Materials.

Ovalbumin. The whites of eggs, which were not more than 24 hours old, were treated according to the methods developed by Hopkins and Pinkus [1898]. The age of the egg is an important factor, since eggs which are more than 36 hours old do not readily yield a crystalline albumin. The ovalbumin was recrystallised twice and then freed from electrolytes by the dialysis of its solution against distilled water, using a good quality of parchment membrane. A stock solution of 5·14 % concentration was prepared but, owing to the denaturation which could not be prevented, this had to be filtered before use, and the protein content determined by estimating the residue after drying to constant weight at 110°. Toluene was used as a preservative.

Gelatin. Coignet's gold leaf gelatin was purified according to the method of Loeb. A stock solution of 1·85 % concentration was prepared, using toluene-saturated water, and was kept as a gel at 5°. The ash of this material was 0·03 % calculated on the weight of dry gelatin.

Irradiation.

Preliminary experiments on the ultra-violet irradiation of ovalbumin indicated that the molecule underwent a profound change: the possibility that this may play an essential part in the formation of pyrenoids of the Protophyta has already been discussed [Howitt, 1930]. The question of the mechanism of this denaturation is being investigated experimentally by one of us. For the present purpose it will be sufficient to describe the production, by ultra-violet irradiation, of the albumin-gold sol which was to be used for electrophoretic experiments. 300 cc. of a solution containing 0·2 % of pure ovalbumin and 0·02 % of gold (as $HAuCl_4$, $4H_2O$) were exposed to a mercury vapour lamp in a quartz flask placed about 5″ from the source of light. A slow stream of nitrogen was passed through the solution with the twofold purpose of excluding atmospheric oxygen and agitating the liquid. The irradiation

was continued for 22 hours in periods of 2–3 hours, in order to avoid excessive heating. A splendid gold sol was formed by this irradiation, a gold mirror also being deposited on the walls of the flask. One volume of this solution was taken for the electrophoretic experiments, 0·2 *N* acetate buffer was added, and the mixture was diluted to 2 volumes, so that finally it contained 0·1 % of protein, and was 0·02 *N* in acetate. It must be pointed out that the product of irradiation of ovalbumin in presence of gold chloride is not necessarily the same as that prepared in absence of gold salt.

Gelatin. A 0·5 % solution was irradiated in a manner similar to that used for ovalbumin, and for 28 hours. A gold sol was then added. The p_H of the solution rose from 4·85 to 6·55. That the protein had altered was shown by the following observations. Precipitation by half-saturated ammonium sulphate was less evident in the irradiated than in the original material. When a 0·1 % solution was allowed to stand in the presence of 0·01 % gold for some weeks, coagulation occurred in certain cases, *viz.*:

p_H	Appearance
2·07	Clear red colour
2·35	,,
2·75	Coagulated, blue
3·38	Purplish red colour
3·56	Light red colour, partly coagulated
5·60	Red colour, clear
7·75	Deep red
8·95	,,

Thus there appears to be a loss of protective power in two zones of p_H. The coagulation at p_H 2·8 was irreversible with respect to gold, while that which occurred up to p_H 4·5 was reversible.

The gold sol was prepared by two methods.

(*a*) Bredig's method, by passing an arc between two gold wires in 0·0005 *N* HCl [Beans and Eastlack, 1915].

(*b*) Phosphorus in ether.

No material difference in velocity due to the different preparations was observed.

Electrophoresis.

Our method, as previously described [1929], consisted in the observation of the motion of a boundary between an acetate buffer (0·02 *N* in total acetate) above and a protein-gold sol below (0·02 *N* in acetic acid or total acetate), which had been brought to the same p_H by the addition of dilute acid or alkali. A potential fall of 222 v. was applied at electrodes of zinc in 5 % zinc chloride and 3 % agar, placed in side-tubes far removed from the boundaries.

The sols contained 0·1 % of protein and 0·01 % of colloidal gold. The concentration of sol must always be kept above that point at which mutual precipitation with the gold occurs. It was shown by Gann [1916] that an acid gold sol prepared according to Donau [1905] is precipitated by gelatin up to concentrations of about 0·0001 % of the latter, and by Freundlich and Loening [1921] that an acid solution containing 0·01 to 0·02 % of gelatin does not

flocculate gold, but that at this concentration the gold still moves electrophoretically towards the positive pole, and therefore behaves as (negative) gold. At higher concentrations, however, 0·05 to 0·10 % gelatin, our results and those of other investigators [Reinders and Bendien, 1928] have shown that the gold migrates to the negative pole, and behaves fully as protein; the velocity changing with change of p_H in the manner which is characteristic of the protein without gold. In alkaline solution it is stated that there is no sensitisation or coagulation of the gold, even when the protein is in very low concentration.

We have also noticed sometimes a coagulation of albumin at or near its isoelectric point in the presence of gold. We consider this to be an entirely different effect from that which takes place in acid solution. The isoelectric effect takes place at high protein concentrations, *i.e.* 1 %. The gold seems to be carried down with the protein and is not changed in colour. Such coagula are easily peptised by a trace of alkali, which is insufficient to make any appreciable alteration in p_H, with the production of an apparently unaltered red sol.

Gelatin lowers the velocity of colloidal gold in all sols and reverses it when $p_H \simeq 4{\cdot}2$ and when the gelatin has a concentration of more than 0·005 % [Reinders and Bendien, 1928].

Irregularities in the motion of protein boundaries "up" and "down" are partly due to differences in potential gradients. The ratios of the conductivities of sols to those of buffers are higher when the sols contain higher concentrations (0·5–1·0 %) of gelatin and albumin than when they contain lower concentrations (0·1 %). Therefore the ratios of the potential gradients are also higher for the more concentrated protein sols. In the case of albumin, the differences between up and down velocities were greatly reduced by correcting for the potential gradient.

Potential gradients may also be set up by diffusion potentials, which were measured in several instances and found to be 9 or 17 mv. in different acid solutions of p_H 3·6. If the diffusion potentials extend over a length of 1 mm. the gradients will be negligible compared with those of the main applied potential, which amount to several hundred millivolts per mm. The ionic diffusing layers will be spread out or made more diffuse when the current is carried by a quicker ion which is following a slower ion, and from regions of lower to those of higher conductivity. In the acid solutions, the boundary on the anode side moves downward, the faster Na^+ following the slower gel^+ cation down, and the faster Cl^- following the slower A^- up. The ionic double layer will on the whole become diffuse after the passage of the current. On the cathode side the ionic double layer will be sharper, the Cl^- ions of the sol moving down and leaving the gel^+ to move up in a region of higher potential gradient, in which also they may be accelerated by the motion of the Na^+ ions. The very high velocities of 3 μ/sec. or more with which the upward boundary sometimes moved may be due to one or other of these causes. On the other

hand, the protein micelles probably carry with them a considerable proportion of the ions which conduct the current, and this will tend to equalise the potential gradients of both boundaries.

These uncertainties seem to be inherent; however we considered that the most important condition is the constancy of p_H throughout the liquid. Differences in p_H not only greatly affect the velocities but also may give much higher diffusion potentials than those observed, which are due to unequal concentrations and mobilities of Na^+, Cl^- and CH_3COO^-. It is possible, while maintaining p_H constant, to equalise also the conductivities in the two solutions by adding a neutral salt such as KCl to one or both. This device was successfully employed by Hardy [1905] who thus equalised the up and down movements of methylene blue. Kruyt [1925] found however that a gelatin sol had different velocities (*a*) when sol and solution were at the same p_H, (*b*) when the conductivities were made the same by addition of neutral salt to the solution. This result was confirmed by special experiments which are described in the series which deals with 1·0 % gelatin.

On account of the possible effect of the addition of neutral salts upon the proteins we have decided not to equalise the conductivities in this way. But, since the correction for potential gradients greatly improves the agreement between the "up" and "down" values, we have determined separately the conductivities of the buffer and sol containing 1 % albumin, and hence have determined the best form in which a mean correction can be made (fourth column of Table I). From the conductivities of the buffer and sol are determined the potential gradients in these parts, as described in the example (below). The means of all the buffer potential gradients are taken, and likewise the mean of all the sols. The mean of these means is used as a divisor of the mean (up and down) velocities at each p_H. The result (fifth column of Table I) agrees in most cases with the values in the fourth column.

Specimen calculations: albumin 1 %. At p_H *4·09.*

$v = +$ 18·5 (down) $-$ 31·5 (up) in mm./hour.

$=$ 5·15 (down) $-$ 8·75 (up) in μ/sec.

κ_1 (sol) and κ_2 (buffer) are proportional to 0·001136 and 0·000538 respectively.

l_1 (sol) $= 20$ cm., l_2 (buffer) $= 34$ cm.

$\frac{E_1}{l_1} = \frac{E\kappa_2}{\kappa_2 l_1 + \kappa_1 l_2} = 2{\cdot}35 =$ potential gradient in sol,

$\frac{E_2}{l_2} = \frac{E\kappa_1}{\kappa_2 l_1 + \kappa_1 l_2} = 5{\cdot}15 =$ potential gradient in buffer,

$v_1 = \frac{v\,(\text{down})}{E_1/l_1} = 2{\cdot}2$, $v_2 = \frac{v\,(\text{up})}{E_2/l_2} = 1{\cdot}7$.

$\frac{v_1+v_2}{2} = 2{\cdot}0$ at $16{\cdot}0\ \frac{2{\cdot}0 \times \eta_{16}}{\eta_{18}} = 2{\cdot}09$ at 18°.

Mean uncorrected "v" $= \frac{5{\cdot}15+8{\cdot}75}{2} = 6{\cdot}79$.

Mean "v" under unit potential gradient at 18° $= \frac{6{\cdot}9 \times \eta_{16}}{3{\cdot}8 \times \eta_{18}} = 1{\cdot}93$.

The agreement is not always so good as this. Thus at p_H 2·90, v_1 (μ/sec.) = 1·67, v_2 = 19·1, E_1/l_1 = 0·83, E_2/l_2 = 6·0. Therefore, at unit potential gradient, v_1 = 1·98, v_2 = 3·2, mean = 2·60 at 17°. In the middle part of the series, from about p_H 4·5 to 8·0, the conductivities of sol and buffer do not differ much from one another, and the "up" and "down" values are in fair agreement, which is usually improved by the correction for potential gradient. There are occasional exceptions such as the velocities at p_H 5·95 which are v_1 = 0·85 and v_2 = 2·0 respectively.

Gelatin 1 % at 28° and p_H *3·63.*

v μ/sec. + electrode 2·5 (down) − electrode 7·65 (up).

κ_1 and κ_2 proportional to $4{\cdot}41 \times 10^{-4}$ and $2{\cdot}26 \times 10^{-4}$ respectively (sol and buffer).

$E_1/l_1 = 2{\cdot}58$, $E_2/l_2 = 5{\cdot}0$.

$v_1 = \frac{2{\cdot}5}{2{\cdot}58} = 0{\cdot}97$, $v_2 = \frac{7{\cdot}65}{5{\cdot}0} = 1{\cdot}53$.

$\frac{v_1+v_2}{2} = 1{\cdot}25$ at $28° \times \frac{\eta_{28}}{\eta_{18}} = \mathit{0{\cdot}99}$ at 18°.

Mean uncorrected v = 5·07.

Mean v under unit potential gradient at $18° = \frac{5{\cdot}07 \times \eta_{28}}{3{\cdot}8 \times \eta_{18}} = \mathit{1{\cdot}05}$.

The correction for viscosity. A series of measurements of the relative viscosities of albumin, gelatin and their mixtures, at various values of p_H, made in the Ostwald viscosimeter, will be the subject of a further communication. Great differences are found between the viscosities of 0·5 % gelatin and albumin on the one hand and 0·5 % and 0·25 % gelatin on the other. Since the velocities of the 1·0 % gelatin and albumin and of the 1·0 and 0·1 % gelatin respectively agree in the main, and a correction on the supposition that the values of "v" are inversely proportional to these viscosity figures, *i.e.* under low rates of shear, would give the curves quite a different character, in the equation

$$v = \frac{1}{4\pi}\frac{HD\zeta}{\eta},$$

it has been assumed that the viscosity η is really that under high shearing stresses, *i.e.* from 400 mm. Hg upwards [Abramson, 1928]. It has also been observed by Freundlich, Abramson and others that the electrophoretic velocities of red blood corpuscles are almost unaffected by great variations in the viscosities of media containing serum, gelatin *etc.*, even by the very high values which are found after gelation has occurred.

Since electrophoresis is due to movements of ionic double layers, it is generally considered that the viscosity is that which affects and varies inversely as the conductivity. It has been found that the addition of a neutral substance, such as starch, in amounts sufficient to produce a very high viscosity has only a slight effect on conductivity [Hardy, 1905].

Since also the temperature coefficient of viscosity is the inverse of and nearly the same as that of conductivity, the correction, *e.g.* to 18°, may be introduced

either as κ_{18}/κ_t or η_t/η_{18}. It is known that the electrophoretic velocities of silver sols, determined at different temperatures, become constant when corrected to a constant temperature [Burton, Physical properties of colloidal solutions]. Abramson [1929] assumes a temperature coefficient of electrophoretic velocity of 2 % per 1°.

We have thought it best to correct the results simply for the alteration in viscosity of water. In our former paper, the velocities were referred to those at 15° as standard. The present series have been referred to 18°, so that the former values are multiplied throughout by the factor η_{15}/η_{18} for comparison.

Table I. *Albumin 1 %.*

p_H	Temperature	v in μ/sec. Mean	$v/3{\cdot}8$ Mean velocity divided by mean potential gradient and corrected to viscosity at 18°	$\frac{v_1 l_1/E_1 + v_2 l_2/E_2}{2}$ Mean of "up" and "down" velocities each divided by its potential gradient and corrected to viscosity at 18°
2·50	18·0°	6·70	+1·76	+2·25
2·90	16·5	10·38	2·83	2·70
3·56	18·5	7·80	2·05	2·56
4·09	16·0	6·90	1·90	2·10
4·44	18·5	4·45	1·16	1·15
5·42	16·0	1·65	−0·45	−0·41
5·95	19·0	4·95	1·26	1·33*
6·48	18·5	4·50	1·17	1·14
6·58	17·0	4·03	1·08	1·02
7·03	19·5	6·40	1·62	1·59
8·24	20·0	6·30	1·57	1·53
9·38	19·5	7·55	1·91	1·92

* Not plotted in Fig. 1.

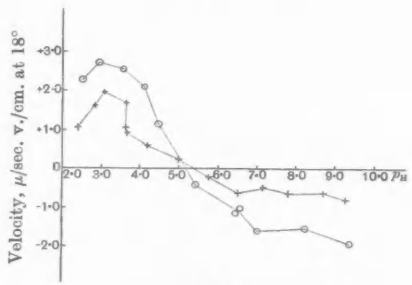

Fig. 1.

—⊖— 1·0 % ovalbumin. —+— 1·0 % gelatin.

Comparison of 1 % gelatin and albumin.

Our previous results, in 0·1 solution, which are included in Table III, seem to show a close agreement between the electrophoresis of the two proteins. A comparison is more difficult in the case of the 1 % solutions on account of the irregularities on the acid side, and the necessity of carrying

Table II. *Gelatin 1 °/₀ at 28°.*

p_H	v in mm./hr. Mean at 28°	"v" in μ/sec. v./cm. at 18°
2·37	19·0	+1·09
2·80	27·5	1·58
3·06	31·7	1·94*
3·63	18·2	1·04
3·63	27·0	1·67†
4·19	9·5	+0·55
4·97	4·0	+0·23
5·77	3·0	−0·19‡
6·48	11·0	0·63
7·15	9·0	0·51
7·83	11·0	0·63
8·70	11·0	0·63
9·30	14·0	0·80

* 1 % gelatin at 25·0.

† 0·8 % gelatin at 25·0. The conductivities of sol and buffer were made nearly the same by the addition of KCl to the buffer.

‡ 1 % at 25°. Both buffer and sol were 0·1 N with respect to KCl, which nearly equalised the conductivities.

Table III. *Gelatin, ovalbumin and their mixtures, 0·1 °/₀ in total protein.*

All velocities expressed as in previous tables.

Albumin 0·1 %				Gelatin 0·1 %				Albumin 0·05 %, gelatin 0·05 %			
p_H	t	v in mm./hr. at $t°$	v in μ/sec. at 18°	p_H	t	v in mm./hr. at $t°$	v in μ/sec. at 18°	p_H	t	v in mm./hr. at $t°$	v in μ/sec. at 18°
2·35	15°	5	$+0·36_5$	2·75	15·0°	+40·0	+3·15	2·77	16·5°	±0·0	0·0
3·56	,,	22	1·61	3·70	15·0	16·2	1·25	3·17	16·5	0·0	0·0
3·95	,,	16	1·17	4·60	15·0	5·0	+0·39	—	—	—	—
4·45	,,	8	+0·58	4·95	16·0	0·0	±0·00	3·90	15·0	+4·0	+0·29
5·56	,,	12	−0·88	5·45	15·0	4·5	−0·35	4·22	19·0	3·5	$0·25_5$
5·95	,,	13	0·95	5·93	17·0	8·2	0·63	4·72	19·0	2·5	0·18
6·55	,,	16·2	1·18	6·60	20·0	8·0	0·55	4·66	16·5	2·0	0·15
7·00	,,	26	1·90	7·15	15·0	6·7	0·52	4·94	16·5	$1·2_5$	0·10
7·65	,,	24·2	1·86	8·02	21·0	10·0	0·67	5·55	15·0	2·8	0·39
				9·27	20·0	11·0	0·86	6·38	15·0	10·0	0·78
								6·98	15·0	8·0	0·63
								7·70	15·0	9·0	0·78
								8·66	16·0	10·5	0·81
								9·55	16·0	10·5	0·81

The velocities in mm./hour are the means of "up" and "down" values, exclusive of those which had diffused. The velocities in μ/sec. are calculated for unit potential gradient.

Table IV. *Albumin 0·1 °/₀ and gelatin 0·1 °/₀ after irradiation.*

Albumin				Gelatin			
p_H	t	v in mm./hr. at $t°$ Mean	v in μ/sec. v./cm. at 18°	p_H	t	v in mm./hr. at $t°$ Mean	v in μ/sec. v./cm. at 18°
2·63	15·0°	15·2	+1·2	2·07	17·0°	38·0	+2·84
3·19	16·0	21·0	1·6	2·35	18·0	37·5	2·74
3·90	17·0	21·2	1·6	2·84	20·0	$32·7_5$	2·27
4·55	17·5	7·8	+0·6	3·38	19·5	19·5	1·37
5·72	15·0	12·5	−1·0	3·56	20·5	$18·2_5$	+1·24
6·30	18·0	15·0	1·1	5·60	20·5	$22·2_5$	−1·52
7·54	19·0	22·5	1·6	7·75	20·0	$22·2_5$	1·54
9·30	19·5	27·7	2·0	8·95	19·5	21·0	1·47
				9·34	19·0	$27·2_5$	1·93

out the gelatin experiments at a higher temperature, 25° or 28°. On the whole it appears that the velocities at each p_H are lower in the case of gelatin. This may be connected with the much higher viscosities of gelatin solutions which we hope to investigate further by another method.

In the case of both proteins, velocities seem to reach maxima at p_H 3·0–3·5, then to fall off, in the case of gelatin, very rapidly, so that they even become zero at still lower p_H values. In this connection it may be noted that the combination curve of 1 % gelatin (p_H plotted against x cc. of HCl added) seems to show that the maximum amount is combined at about p_H 2·5, but the acid blank correction is large here so that the amount actually combined is rather uncertain [Atkin and Douglas, 1924].

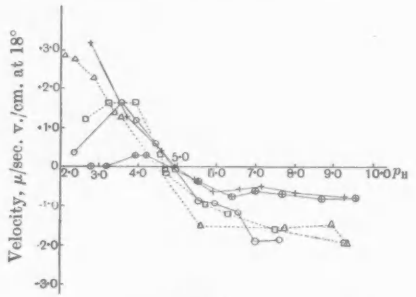

Fig. 2.

⊖ 0·1 % ovalbumin. □ 0·1 % irrad. ovalbumin.
\+ 0·1 % gelatin. △ 0·1 % „ gelatin.
⊕ 0·05 % ovalbumin. 0·05 % gelatin.

The isoelectric point of gelatin determined graphically seems to lie above p_H 5·0. On account however of the low values of $\Delta v/\Delta p_H$ on either side of this point, a small alteration in the somewhat irregular velocities of positively charged gelatin would make a large difference in the point at which the line cuts the axis of zero velocity.

On the alkaline side, the velocities "up" and "down" are in good agreement, and as previously found in the case of 0·1 % solutions, become nearly constant at about 0·5–0·7 μ/sec. between p_H 6·0 and 6·5. Referring again to the combination curve of gelatin we find, after p_H 6·0, a rapid increase of p_H with added alkali, showing that the formation of alkali gelatinate, or, according to the newer view, the displacement of the weaker set of basic groups from combination with OH, has reached a maximum. Therefore, no additional charge on the micelle is to be expected until higher values of p_H are reached. In the curve of 1 % gelatin [Atkin and Douglas, 1924], buffer action increases again at about p_H 9·5. This is also about the point where in our results an increase in negative velocity reappears.

In the case of 0·1 % solutions of albumin, the negative velocities appear

to increase with increasing p_H at about the same rate as that at which the positive velocities increase with diminishing p_H. The velocities on the alkaline side were more irregular in the case of the 1·0 % solutions. They increased with p_H up to 1·0 μ/sec. or more, at p_H 6, and at higher values of p_H, continued to increase, although more slowly. They remained considerably above the velocities of 1 % gelatin at the same p_H.

Mixtures of 0·05 % gelatin and 0·05 % albumin.

The mixtures contained as usual 0·01 % of gold sol and were adjusted by addition of acid and alkali to the p_H of the 0·02N acetate buffers. The results in Table III are compared with those of the 0·1 % proteins measured separately. It might be expected that the motion would be intermediate between those of the separate proteins, or that it would be the same as that which has the greater velocity at each p_H, since each protein should carry with it sufficient gold to make the margin visible. Neither expectation was realised.

On the alkaline side, the protein behaves almost exactly like gelatin, which has the lower velocity. The tendency of the albumin to combine with increasing OH^- concentrations, giving an increasingly negative micelle, is somehow masked by the gelatin, which hardly increases its negative charge in this range of p_H.

On the acid side, the mixture does not behave like either protein. The velocities become somewhat irregular—a negative value having been registered at the isoelectric point 4·7, which according to our results in 0·1 % solution should have given not a zero, but a positive velocity. At p_H 4·0 a slight positive velocity was noted, but at lower p_H values the velocities fell to zero. It appears as if the mixture forms a more stable amphoteric ion, which is not appreciably opened up by the addition of acids. The viscosities of the mixtures, which will be the subject of a further communication, are to some extent intermediate but nearer to those of albumin.

The irradiation does not appear to have made any material alteration in the electrophoretic velocities of either protein. On account of the coagulation, velocities cannot be observed so close to the isoelectric point of the irradiated gelatin. A small portion has undoubtedly been rendered extremely sensitive to oxidation by the gold chloride, and has been destroyed, but the remainder seems to retain the same positive or negative charges.

SUMMARY.

1. The mean velocities of albumin are higher than those of gelatin both in acid and in alkaline solutions.

2. Neither the curve of gelatin nor that of albumin is symmetrical on the acid and alkaline side of the isoelectric point.

3. On the acid side the velocities increase rapidly with diminishing p_H to a maximum, and then fall off.

4. On the alkaline side the velocities increase up to p_H 6–7, then fall off and reach a constant or nearly constant value, which is lower and more nearly constant in the case of gelatin.

5. Mixtures of gelatin and albumin behave anomalously.

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